Human herpesvirus-6 and the cytokine network in human glial cells

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HUMAN HERPESVIRUS-6 AND THE CYTOKINE NETWORK IN HUMAN GLIAL CELLS

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Contents

Abbreviations

APC	antigen presenting cell
BBB	blood brain barrier
BDNF	brain-derived neurotrophic factor
BMP	bone morphogenetic protein
CNS	central nervous system
CPE	cytopathological effect
CSF	cerebral spinal fluid or colony stimulating factor
CV	coefficient of variation
EAE	experimental autoimmune encephalomyelitis
EBV	epstein-Barr virus
FCS	foetal calf serum
GFAP	glial fibrillary acidic protein
GM-CSF	granulocyte-macrophage colony-stimulating factor
HHV-6	human herpesvirus-6
HLA	human leucocyte antigen
LPS	lipopolysacharide
MAG	myelin associated glycoprotein
MBP	myelin basic protein
MOG	myelin oligidendrocyte glycoprotein
MRI	magnetic resonance imaging
mRNA	messenger RNA
MS	multiple sclerosis
NBB	Netherlands brain bank
NO	nitric oxide
NT	neurotrophin
PBMC	peripheral blood mononuclear cells
PLP	proteolipid protein
PMA	phorbol myristate acetate
PP	primary-progressive
PR	progressive-relapsing
ROS	reactive oxygen species
RR	relapsing-remitting
SP	secondary-progressive
Th	T helper
TLR	toll like receptors

CHAPTER 1

General Introduction

Multiple sclerosis, a general introduction

Multiple Sclerosis (MS) is the most common neurodegenerative disease among young adults in the Western world affecting about 1 per 1,000. In healthy individuals most nerves in the central nervous system (CNS) are insulated by a fatty substance called myelin, which permits the efficient transmission of electrical impulses signaled by the nerve cell. During MS the local occurrence of inflammatory reactions in the CNS white matter leads to focal areas of demyelination (lesions or plaques). The insulating myelin that is wrapped around axons is degraded. A first attempt to separate MS from other neurological diseases was made by Charcot over a hundred years ago. Charcot described the pathology of MS in terms of demyelination and relative sparing of axons. Nowadays it is known that inflammatory demyelination and the production of nitric oxide (NO) result in conduction block and axonal damage and eventually in transection of axons and axonal loss during disease progression (1). Axonal loss is the pathological hallmark of MS and increases in lesions as they age. As a consequence of demyelination and axonal loss neuronal signal induction is impaired giving rise to many different neurological symptoms. There are two different phases of axon degeneration, the first occurring during active myelin breakdown and the second in chronic demyelinated plaques in which the naked axons appear to be more susceptible to further damage. Although myelin can normally repair itself and remyelination has been seen in up to 70 % of lesions, scarring by the influx and proliferation of astrocytes (astrogliosis) happens too rapidly for complete healing to take place. Repeated inflammation of the nerves results in many (Multiple) lesions which evolve into dysfunctional scars (Sclerosis). Scars are visible macroscopically in the CNS of patients upon obduction. MS is often chronic and progressive due to repetitive inflammatory reactions.

Clinical and pathological features of MS

A person may live for years with MS before a diagnosis is set. The initial attack of MS occurring sometimes as early as the teen-age years may be brief and mild, and may not even be recognized. Generally the first major attack, lasting for weeks or months, takes place between the age of 20 and 40, and further attacks follow at erratic intervals. The symptoms temporarily abate or disappear for unknown reasons, but recurrence is highly likely although usually after a long latency period. The first attack is followed by a period of remission of perhaps years before the next episode. The symptoms of the disease are diverse, depending on where in the brain, spinal cord or optic nerve these patches of demyelination and axonal damage are located.

Symptoms range from relatively minor physical annoyances to major disabilities such as loss of muscular coordination, stiffness (spasticity), impaired vision or even loss of vision, bowel or bladder incontinence and general tiredness or extreme exhaustion. As a result of the lasting CNS damage, 77 % of MS patients are limited to some degree in their activities and about 25 % become wheelchair-bound.

There are four clinical subgroups of MS. Relapsing-remitting (RR) MS is the most common one, following a relapsing-remitting course with transient neurological deficits that tend to resolve completely at the beginning of the disease. With time RR-MS may change into a more progressive subtype; secondary-progressive (SP) MS which is characterized by incomplete recovery or steady progression of the disease. Patients with the primary-progressive (PP) MS subtype have an overall poorer prognosis. They do not experience an initial attack since the disease progresses from onset, with occasional plateaus with an absence of relapsing-remitting periods. Progressive disease from onset but associated with clear acute relapses followed by full or partial recovery is called progressive-relapsing (PR) MS. Periods between relapses are characterized by continuing progression. Only 5 % of MS patients have this very rare clinical PR course. Sometimes exacerbations in MS patients are preceded by special events like physical exercise, mental tension or a bacterial or viral infection, but most of the time they appear without any traceable direct cause. The clinical course of MS is rather unpredictable. To be certain of the diagnosis of MS different clinical and laboratory tests are necessary including electrophysiological tests, magnetic resonance imaging (MRI) of the brain and the spinal cord as well as analysis of the cerebrospinal fluid (CSF) for the presence of oligoclonal immunoglobulins (2). Since many MRI signals are non-specific to MS reliance on these data alone is not sufficient for the diagnosis and can lead to diagnostic errors. This becomes an important issue in epidemiological surveys and participation in clinical trials using very expensive disease modifying drugs. Confirming the diagnosis signs and symptoms have to be present disseminated in time and space. Episodes of symptoms have to last for 24 h and must be separated by at least one month. Signs of MS must be attributable to involvement of at least two parts of the brain or spinal cord (3). MS neuropathology may appear in any CNS region and even in grey matter, but some areas are more frequently affected including perivascular white matter, the periphery of cerebral gyri, optic nerve, chiasm, pons, cerebellar peduncles, medulla oblongata and the spinal cord (4,5). Furthermore, there is a high incidence of active lesions in the hypothalamus which might form the basis for autonomic and endocrine alterations in MS patients (6).

There are different forms of lesions including pre-active, active, chronic active and chronic inactive lesions. These different lesion stages are suggested to reflect stages in disease progression (7). The two most common lesion types are chronic active and chronic inactive lesions. In chronic active lesions the number of T-cells and macrophages increase from the center to the edge of the lesion and penetrate white matter adjacent to the lesion. Inflammatory reactions are characterized by foci of myelin degeneration. In inactive chronic lesions T-cells and macrophages are fewer and more demyelination is seen. The age of the lesion can be established by the presence of myelin degradation products in macrophages (8).

Lesions of different MS patients show a profound heterogeneity in the structural and immunopathological patterns of demyelination and oligodendrocyte pathology (9). However, for a given individual, multiple lesions show the same pattern regardless of the localization or the stage of the lesion. Lucchinetti et al. investigated brain samples from biopsies (a rare procedure sometimes necessary to exclude other possible diseases) as well as from post mortem MS brain (10). Four distinct patterns of demyelination are detected based on myelin damage and the presence of immune cells. This finding suggests that MS is a group of similar clinical diseases that have different causes and involvement of different myelin destruction mechanisms. In contrast to these findings Barnett et al. showed that heterogeneity may also exist among lesions in a single patient (11). Some lesions show the involvement of antibodies in the demyelination process while others show more evidence for a primary oligodendrocyte defect (e.g. viral infection) (12). Different lesion types among patients or within a single patient might be due to individual disease progression, age of disease as well as perhaps to infiltrated T-cells reacting against different antigens (13).

Remyelination in MS lesions is promoted by neurotrophic factors and growth factors. Various experimental models of demyelination and remyelination have shown an increased presence of growth factors including insulin-like growth factor I (IGFI), fibroblast growth factor 1 (FGF-1), FGF-2, platelet-derived growth factor A (PDGF-A), TGF-β1, nerve growth factor (NGF) and ciliary neurotrophic factor (CNTF). Increased production of NGF and other trophic factors can suppress inflammation by switching the immune response to an anti-inflammatory, suppressive mode in a brain-specific environment (14). Growth factors are expressed during the early stages of remyelination and promote recruitment, proliferation and motility of adult oligodendrocyte progenitors (OP). The presence of these adult OP in lesions is necessary to start remyelination. Growth factors and neurotrophins play a role in orchestrating the behavior and survival of the adult OP in remyelination and may be used as a

therapeutic approach to promote remyelination in clinical disease (15). A clearer path to achieve therapeutic remyelination might emerge from careful dissection of the roles performed by growth factors and other key mediators of this important repair process in MS.

MS Therapies

MS is associated with enormous direct and indirect costs for the patient, the health care system and society. The patients carry most of the economic burden of the disease including earnings loss and equipment needed in normal daily life. Results of cost-effectiveness studies provide a basis for analyses of new therapeutic interventions for MS (16).

There is currently no cure available for MS and most of the therapies are based on drugs that suppress the immune response in a non-antigen specific manner. Suppression of the inflammatory process and restoration and protection of glial and neuron functions are the major subjects in MS treatment. Glucocorticoids are widely used in the treatment of a variety of autoimmune diseases because of their anti-inflammatory and immunosuppressive properties. Synthetic glucocorticoids including prednisone and methylprednisone are given during severe relapses in RR MS patients. Several immunosuppressants including azathioprine and cyclophosphamide show beneficial effects in terms of reducing exacerbations and clinical progression (17). However, all of these drugs have serious side-effects when used for a longer period of time. Treatment with interferon-β (IFN-β) results in 30 % reduction of the relapse rate and the lesion frequency on MRI is decreased in RR MS patients. IFN-β treatment is also effective in SP MS patients (18). IFN-β probably acts through immunomodulatory rather than immunosuppressive effects. Copaxone is an alternative drug that appears to have both immunological and potential neuroprotective effects along with a favorable side effect profile, similar to that of IFN-β. Its efficacy is stable or may even increase in time (19). Furthermore, various experimental treatments for MS are evaluated in small clinical trials (20). However, the mechanisms by which these drugs affect MS progression are unknown.

Genetic, environmental and hormonal factors

The etiology of MS remains largely unknown despite intensive scientific and clinical research. The involvement of genetic and environmental factors in the initiation and progression of the disease is generally accepted. MS is not only unpredictable in its on-again, off-again patterns and its broad symptom spectrum it also strikes the population in uneven ways. Women

are twice as susceptible as men and the disease is twice as common among Caucasians as among African-Americans (21,22). Although MS is found in Japan, China and some other temperate, eastern countries, it is much rarer than it is in the West. The incidence of MS is even higher in northerly regions: the frequency of MS in Canada for example is twice that of the United States. Risks for first degree relatives are increased by 20 fold as compared to the general population. Twin studies show that in 26 % of monozygotic twins both twin pairs are affected as compared to 4 % of dizygotic twins and 1.9 % of non-twin siblings with MS (23). The genetic influence on the susceptibility to MS is modulated by several genes. Certain human leukocyte antigen (HLA)-class I genes are weakly associated with MS (HLA-A3 and HLA-B7) while HLA-class II genes show stronger association. HLA-DR associations may differ in certain geographically distinct populations. Association with MS is found for HLA-DR4 (Italian and Arab population), HLA-DR6 (Mexican and Japanese population), HLA-DR2 and HLA-DR15 (Caucasian population) as well as for HLA-DQ genes (24). Studies have also revealed a weak association of other genes of the HLA gene cluster on chromosome 6 with MS (25).

Studies on the genetic susceptibility for MS also include establishment of MS-associated gene sequence polymorphisms. Many polymorphisms have presently been associated with MS. Recent MS-associated polymorphisms include for example those in the matrix metalloproteinase (MMP)-9 gene, in pigmentation related genes, in the CCL2 promoter, in the CTLA-4 gene as well as in the FAS-640 gene and αB-crystallin (26,27,28,29,30). A CD95 polymorphism associated with MS is found to be restricted to women which is in line with their higher susceptibility to this disease (31). These polymorphisms appear to suggest that the immune system is involved in MS since most of these genes involved are immunologically relevant. However, it should be taken into account that this bias might be due to specific selection of immunologically relevant genes for these studies. Also, comparing all information on polymorphisms linked to MS reveals that there are much less data on unlinked polymorphisms, consistent with a publication bias. Despite genome wide mapping it is still not clear which genes are involved in the pathogenesis of MS.

Geographic differences in prevalence may be explained by genetic make-up. However, they have also been attributed to nutrition and Ultraviolet (UV) dose. Sunlight and especially vitamin D3 are suggested as environmental factors in MS disease (32,33). High vitamin D levels are associated with low lesion activity in RR and SP MS patients and *vice versa* (34). The nadir in vitamin D supplies correlates with both the peak of MS lesions activity in German MS patients and the peak of MS disease onset in Switzerland

(35,36). Furthermore individuals who had the highest residential and occupational sunlight exposure were shown to have a lower mortality risk from MS (37). The beneficial effects of sunlight exposure are independent of country, origin, age, sex and socioeconomic status. These findings on the association of sunlight and vitamin D with MS might help explain why the incidence of this disease around the equator is less as compared to northern Europe. Dietary factors including fish might increase vitamin D production. Furthermore fish contains omega-3 fatty acids, which are the components of anti-inflammatory eicosanoids belonging to a family of hormone-like compounds, as well as polyunsaturated fatty acids (PUFAS). Distribution of MS cases in Western Norway showed a higher prevalence in the inland areas as compared to coastal areas (38). This distribution has been suggested to be due to a larger consumption of fish in coastal areas. Nordvik et al. reported that daily consumption of fish oil (omega-3 fatty acids) together with vitamins improved the disease outcome of newly diagnosed MS patients (39). Depletion of fatty acids, zinc, magnesium, selenium and vitamins has been correlated with trapped NO and superoxide resulting in the free radical peroxinitrite which induces myelin damage. Menstruation cycles correlate with depletion of these components which might help explain the higher incidence of MS in women (40). Furthermore, the increased risk of MS in women is believed to depend in part on the influence of sex hormones on the immune system. The mechanism of estrogen-induced immune suppression is suggested to play a role (41). The rate of relapses declines in women with MS during their pregnancy and especially during the third trimester. On the other hand the rate of relapses increases during the first three months post partum before returning to the pre-pregnancy rate (42,43). Stressful events such as illness, problems with close family members, job stress or financial problems are related to the increased occurrence of exacerbations in RR MS patients (44). Although certain types of psychological stress suppress immune reactions the impact of these stressful events was independent of the triggering effect of infections on exacerbations. The mechanism behind stressful events and increased exacerbations is still unclear. Modulation of the hypothalamic-pituitary-adrenal axis and the sympathetic system might explain its impact on inflammatory reactions in MS pathogenesis.

The role of the immune system in MS

The human CNS has for a long time been considered an immunologically privileged site because of the existence of skull bones, the CSF, the meninges and the blood brain barrier (BBB). The existence of a BBB restricts immune cell migration and soluble molecule diffusion from the

systemic system into the CNS (45). Furthermore, presumed lack of lymphatic drainage in the CNS as well as its inability to reject allografts appeared to support this idea of immunological privilege (46). However, it has become increasingly clear that an activated BBB promotes the influx of leukocytes and that (activated) $CD4^+$ and $CD8^+$ T-cells can enter the CNS by passing the impaired BBB (47). Infiltrated T-cells are involved in regulation of immune responses, inflammation and ultimately repair during a variety of CNS diseases. They produce many immunoregulatory mediators such as cytokines, chemokines and growth factors (48). Different types of $CD4^+$ T-cells are involved and can be distinguished by their cytokine production. T-cell subtypes include for example Th(helper) cells and regulatory T-cells (Treg). These different T-cell types regulate immunity to infection and self-tolerance (49). The various T-cell types express different cytokine profiles. Th1 cells for example tend to produce pro-inflammatory cytokines while Th2 cells tend to produce anti-inflammatory cytokines. A complex network of interactions between microglia, astrocytes and T-cells is involved in determining the balance between Th1 and Th2 cells (50). Immunohistochemical studies have shown that MS lesions are characterized by perivascular infiltrates of T-cells, B-cells and macrophages. Some of these infiltrating T-cells are activated (51). The inflammatory reaction in the CNS during MS is accompanied by the production of many inflammatory mediators. Infiltrating T-cells express cytokines notably including the pro-inflammatory cytokine IFN-γ, chemokines, MMPs, NO and reactive oxygen species. These mediators stimulate a variety of physiological, neuroendocrine and behavioral responses of the CNS. The CNS, in turn, regulates the immune system via systemic and local routes including neuroendocrine pathways and the autonomic and peripheral nervous system (52). Figure 1.1 shows the cells and reactions which are involved in MS disease and lesion development. The fact that activated T-cells are clearly present in MS lesions but have not been found in the periphery at levels different from those found in healthy controls suggests that their antigen is primarily recognized within the CNS (53). The autoantigens relevant to MS are generally believed to reside in myelin. The most abundant proteins in myelin are proteolipid protein (PLP) that accounts for 50 % of the total protein mass in myelin and myelin basic protein (MBP) that accounts for 10 to 15 % of all proteins. Minor myelin proteins include myelin-associated glycoprotein (MAG), myelin oligodendrocyte glycoprotein (MOG) and 2′, 3′-cyclic nucleotide 3′-phosphohydrolase (CNPase). Comparing myelin from healthy controls to that from MS patients indicates that MS-affected myelin contains higher levels of αB-crystallin (54).

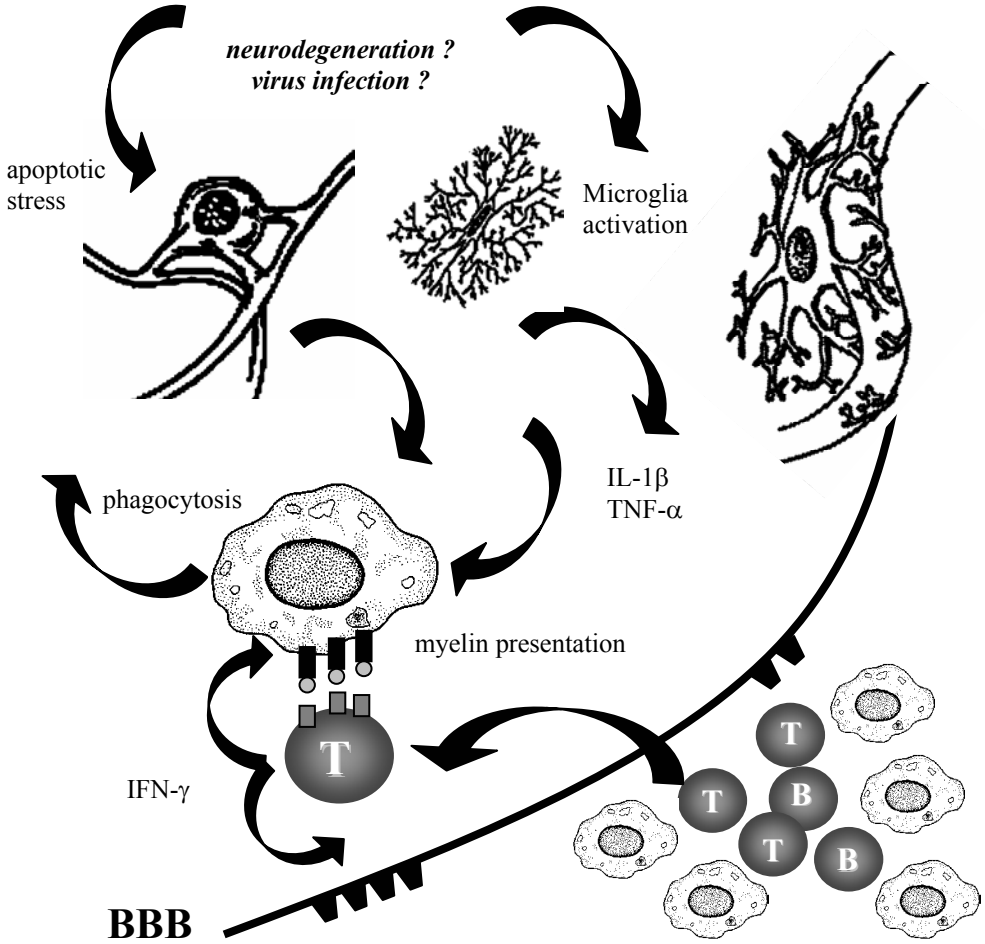

Figure 1.1. Likely events in lesion development during multiple sclerosis
The first event in lesion development probably involves a local endogenous insult in the CNS of unknown origin that leads to apoptotic stress in oligodendrocytes and activates microglia either directly or indirectly. Activated microglia develop into phagocytic macrophages that begin to sample myelin. At the same time they will activate the blood-brain barrier (BBB) to some extent by production of IL-1β and TNF-α. This will promote non-specific leukocyte infiltration and leads to a mild inflammatory reaction. When, however, sufficient numbers of myelin-reactive memory T-cells are contained in the emerging infiltrate this initially mild reaction may 'explode' by myelin-triggered production of IFN-γ. This will strongly amplify phagocytosis and infiltration by yet more leukocytes. A demyelinating lesion develops.
Note that lesion development is likely driven by events within the CNS itself, and not by the peripheral immune system that –instead- acts simply in response to these events. Microglia and astrocytes are important regulators of the local inflammatory process.

Interestingly, human peripheral blood T-cell responses to the complete collection of myelin from MS patients are dominated by the specific response against αB-crystallin. The absence of αB-crystallin from lymphoid tissues in humans, allowing autoimmune responsiveness to develop may account for this (55). This responsiveness appears to be unique to humans since several other mammalian species examined showed expression of αB-crystallin in secondary lymphoid tissues and even in the thymus, and these species are characterized by profound tolerance for self αB-crystallin. As this is clearly different in humans, the protein is therefore considered as a candidate autoantigen in multiple sclerosis (56).
An animal model for inflammatory CNS demyelination is experimental autoimmune encephalomyelitis (EAE). This model, most frequently studied in rodents or non-human primates, is in line with an autoimmune etiology of MS. EAE is induced by active immunization with myelin-derived antigens in a powerful adjuvant or by transfer of myelin specific T-cells (57,58). EAE is thus based on introduction or activation of peripheral T-cells against myelin antigens resulting in CNS infiltrates and myelin damage.

Viral associations with MS

Many epidemiological, clinical and neuropathological data are suggestive for an association between MS and viral infections. A viral infection has long been suggested to play a role in MS largely because most other demyelinating CNS disorders of known origin are virus induced. Also, animal model data confirm that a virus could in principle trigger disease with characteristics similar to MS. Active infection of mice with for example the neurotrophic picornavirus Theiler´s murine encephalomyelitis virus (TMEV) or with Semliki Forest virus (SFV) results in inflammatory demyelination. Furthermore, the beneficial effects of treatment with the anti-viral agent IFN-β also supports involvement of viral infection in the pathology of MS (59). It has been well established that viral or bacterial infections may trigger MS exacerbations, once the disease has manifests itself, as reported for upper airway infections which might be caused by influenza virus (60,61). Exacerbations with onset around the time of clinical infections result in more sustained neurological damage as compared to non-infection-associated exacerbations (62). However, there is no indication that this sustained damage is associated with enhanced opening of the BBB.
Many viruses have been proposed as a potential causative agent in MS (Table 1.1).

Table 1.1. Viruses during the years associated with multiple sclerosis

Virus	Reference
Rabies virus	63
Parainfluenza virus	64
Herpes simplex virus	65
Measles virus	66
Varicella zoster	65
Mumps virus	67
Rubella	67
Canine distemper virus	68
SMON-like virus	69
Tick borne encephalitis	70
Epstein-Barr virus (EBV)	71
human T-lymphotropic virus type I (HTLV-I)	72
Simian virus 5	73
LM7 (retroviruses)	74
Human herpesvirus-6 (HHV-6)	75
MS-associated retrovirus (MSRV)	76
Polyomavirus (JCV)	77
Human endogenous retroviruses (HERV)	78
Human coronavirus	79
morbillivirus	80
Picornavirus	81
Chickenpox virus	82

Yet, a role of viral infection as a causative factor in MS remains to be established. Viruses can, especially upon reactivation, interfere at various levels with antigen presentation and co-stimulation functions as well as with cytokine and chemokine signaling. Such effects could provide a basis for understanding how even ubiquitous viruses could play a role in the pathogenesis of MS or other neurological diseases and CNS inflammation. For no single virus, a conclusive and specific association with MS has been found so far. Recently, childhood infections, occurring at the age of ≤ 7 years, including measles, chickenpox, rubella, whooping cough and mumps were reported to be significantly higher in MS patients than in controls (82). To make it even more complex bacterial pathogens such as Chlamydia pneumonia have also been associated with MS (83).

The association between MS and Epstein-Barr virus (EBV) in particular has attracted much attention, and this viral association can be considered the strongest known. The most striking findings so far (84) were that essentially all MS patients are EBV seropositive versus 90-95 % of the general population and that anti EBV immunoglobulin (Ig) serum titers are usually

higher in MS patients (85). Also a delayed primary infection with EBV resulting in a history of infectious mononucleosis is clearly associated with an increased risk of MS later on in life (84,86). Intriguing is the fact that EBV infection in B-cells enhances the expression of αB-crystallin, the above-mentioned candidate autoantigen in CNS myelin of MS patients (54). Presentation of αB-crystallin by major histocompatibility complex (MHC) class II molecules in EBV-infected human B-cells to T-cells leads to activation of pro-inflammatory αB-crystallin-specific Th cells (55). In this way a memory T-cell pool against αB-crystallin could be established as a side effect of EBV infection. Later on in life, these memory T-cells could help trigger an 'explosive' inflammatory reaction in the CNS when another event would induce BBB impairment and oligodendroglial stress (56). Migrant studies appear to be consistent with a role of EBV as a necessary co-factor in MS. Most studies among migrant populations that relate alterations in the risk to developing MS to the age of migration suggest that the critical age is before puberty (the age of 15) (87). Moving from high-prevalence areas to low-prevalence areas shows that these migrants attain the lower risk if they move before puberty but retain the high risk status if they migrate after puberty. For example Dean and Elian have reported that immigrants to England from India and Pakistan at an age younger than 15 years had a higher risk of developing MS than those immigrating after that age (88). Data from a study among North African immigrants to France show that their onset of MS is at an earlier age as compared to French-born patients. This suggests that MS is primarily an environmental disease acquired after childhood (89). These data indicate that there is a co-factor before puberty mediating the risk to acquire MS. Young children in high risk areas are not affected (yet) by this co-factor, consistent with the idea that this co-factor could include delayed primary infection with EBV. Yet, not all studies are equally clear on this subject (90,91). For example, a 75 % reduction was seen in the risk to develop MS among British and Irish immigrants in Australia as compared to the risk of their native countrymen (92). The reduction affected both adult and child immigrants.

The association between HHV-6 and MS

Apart from EBV that can be considered the strongest viral link in MS, several observations have also been reported during the years that point to an association of human herpesvirus-6 (HHV-6) with MS. However, a distinct controversy exists as to the possible involvement of this virus in MS (93,94). Studies that associate HHV-6 with MS have for example revealed HHV-6 nuclear staining in oligodendrocytes and neurons from MS patients

but not in material from control donors (95). HHV-6 infection has been found more in MS patients as compared to healthy controls and in more active plaques than in inactive ones (96,97). Furthermore, active HHV-6 infections have been reported to be more frequent in lymphoid tissues, peripheral blood lymphocytes, serum and urine of MS patients as compared to these tissues from healthy control donors (98,99,100,101). Finally, HHV-6 antibody titers have been reported to be elevated in serum and CSF from MS patients as compared to serum and CSF from healthy controls (75,99,102). On the other hand several other studies have failed to produce evidence in support of a specific association of HHV-6 with MS (103,104,105,106,107,108). These studies show that reactivities to HHV-6 and levels of HHV-6 DNA in serum and CSF were similar in MS patients as compared to healthy controls. Furthermore, Rodriguez et al. reported no signs of HHV-6 DNA in CSF from MS patients (109). Moore and co-workers compared 28 studies on the association between HHV-6 and MS (111). In their review they concluded that although many studies prove evidence for a relationship between HHV-6 and MS none of the studies showed a causative association.The conflicting results on a possible association between HHV-6 and MS do not necessarily rule out a role of HHV-6. Negative results may be due to different population studies, different sensitivities of the technique used, or a different DNA sequence target for PCR amplification. The existence of two different variants of HHV-6 may also account for discrepant results (110) and also, HHV-6 may not be relevant to all stages in MS or all stages in lesion development.
A major challenge in further clarifying a possible association between HHV-6 and MS is identification of molecular mechanisms by which the virus could influence cell-biological processes that are specifically relevant to MS. While for EBV such mechanisms have been suggested (see below), no data or models are currently available to indicate how HHV-6 could influence either T-cell functions, CNS pathways or other processes in ways that would be consistent with a presumed role as etiological agent in MS.

Ways for viruses to influence autoimmunity

Many mechanisms have been suggested by which a virus infection can lead to an autoimmune response against myelin, as is supposed to play a crucial role in MS (112). The most straightforward way for a virus to influence MS would be viral infection of the CNS itself. This could initiate a virus-specific inflammatory response resulting in demyelination as bystander damage without necessarily involving an autoimmune response. The pathology of MS lesions, largely reflecting a classic host defense response, would be consistent with this theory, if it would not be for one crucial issue:

a virus specifically associated with active MS has not been identified yet. It has been argued that a powerful host defense response against a virus could possibly eliminate the pathogen before it could ever be detected in the resulting lesion (113). Such a 'hit-and-run' model is clearly difficult to falsify. In the absence of a well-established causative agent in MS, many attempts have been made to explain the development of MS lesions based on a virus-associated autoimmune etiology, a line of thinking that is strongly supported by animal model data.

First, cross-reactivity between viral antigens and normal myelin components continues to be a popular theory to explain how viral (or bacterial) infection could activate autoimmune T-cells. In this model of so-called molecular mimicry viral infection does not necessarily need to take place in the CNS since structural similarity between pathogen-specific determinants and myelin determinants could cause pathogen-activated T-cells to cross react against myelin. Even when activated elsewhere in the body, such activated T-cells could cross the BBB and set off an autoimmune reaction in the CNS similar to what happens during EAE. Examples of such cross reactions between pathogens and self antigens have been documented using individual T-cell clones but so far, relevant cross-reactivity between pathogens and myelin components at the level of polyclonal responses remain to be demonstrated. Also, disease-initiating peripheral T-cell reactivity against myelin components in MS patients different from what can routinely found also in healthy controls remains to be established, while being a crucial element in this model of molecular mimicry.

Secondly, it has been suggested that a first inflammatory insult caused by either a virus or a bacterium could promote subsequent development of waves of novel T-cell reactivities to locally released self antigens. In this scenario of 'determinant spreading', such newly generated responses would be largely autoimmune and able to sustain a chronic process of (more or less self-perpetuating) autoimmune disease. A major problem with this model, however, is the question how such a cascade of spreading responses is to be reconciled with organ specificity as seen in MS. After all, most of the locally released antigens are also expressed in several other tissues. In addition, no evidence has been found so far for any of such spreading responses to be detectable in MS patients, nor has any association been found between MS and non-specific inflammatory CNS damage such as occurs in brain trauma that would similarly be expected to trigger determinant spreading (114).

While still other ideas have been put forward, one final alternative model that combines an autoimmune mechanism and viral infection in yet another way centers around αB-crystallin. As stated before, EBV infection of B

cells (and perhaps infection by some other viruses as well) is known to induce *de novo* expression of αB-crystallin in infected cells, leading to its presentation to helper T-cells. Since in humans this occurs in the pro-inflammatory environment of active viral infection, and against the background of lack of central tolerance, a T-cell response will be mounted against αB-crystallin along with that against viral determinants. While not the driving force for any autoimmune attack at the time of infection, the autoimmune repertoire will persist for life since the triggering virus does so too. When later on, oligodendroglial stress for whatever reason occurs, these αB-crystallin-specific memory T-cells may find their antigen in extracellular myelin of the CNS again, should they find themselves non-specifically recruited by an activated BBB. In this model, the induction of the memory repertoire against a myelin antigen by the virus is not a trigger but a necessary co-factor in the development of MS. The association between EBV and MS reflects this.
However intriguing some of the above models may be, none of the above mechanisms have been conclusively proven to play any role in MS so far. While the almost intuitive conviction remains that viral infection is very likely to play a role in MS, more data are required to illustrate how certain viruses can impact on the cells and mediators that control lesion formation in MS. Clearly, some of the key players are to be found in the CNS itself and they include glial cells that are at the very heart of MS in regulating much of the local inflammatory reactions.

The function of astrocytes and microglia in regulating inflammation and repair in the CNS

The human CNS contains two distinct classes of resident cells: neuronal cells and glial cells. Glial cells are classified as macroglia and microglia. Macroglia can be divided again into oligodendrocytes and astrocytes. Oligodendrocytes are responsible for the production of large membrane sheaths that are wrapped around axons forming an insulating layer, the myelin sheath. Glial cells have long been thought to perform only maintenance roles in the CNS providing trophic factors to neurons, supporting the BBB, maintaining a healthy balance of ions in the brain, and eliminating invading pathogens. Nowadays, it is known that glial cells fulfill many more functions. They are far better equipped to play an active role in immunological processes as compared to neurons (115). By producing IL-10, TGF-β and apoptosis-inducing factors, astrocytes form a barrier against expansion of pro-inflammatory T-cells and thus limit local inflammation (116). They actively regulate BBB functioning and control the structural and

functional plasticity of synapses in developing and adult CNS (117). Astrocytes also play a role in cell-cell communication through gap junctions, and influence neuronal signaling, neuroendocrine interactions and even behavioral responses (52). Microglia on the other hand play key roles in immune-mediated events in the CNS as resident macrophages but are also crucial in repair en regeneration.

Astrocytes and the control of inflammation

Astrocytes are the largest and most abundantly present glial cell type in the CNS. They are coupled by gap junctions that are composed of intracellular channels permeable to ions and small molecules. Junctional channels and connexins (their molecular constituents) provide the basis for an efficient and direct intercellular communication in astrocytic networks (118). Astrocytes play a key role in BBB maintenance, control of inflammatory responses, the formation of neuronal networks, development, homeostasis, repair and signaling networks in the CNS by producing a wide variety of cytokines, chemokines, growth factors and by expressing receptors for these molecules. Under inflammatory or otherwise pathological conditions astrocytes become activated and display enhanced production of several cytokines, chemokines and growth factors. Also, astrocytes generally start to proliferate and migrate under such conditions. Reactive astrocytes show marked induction of glial fibrillary acidic protein (GFAP) and accumulate at the border between intact and damaged tissue, a phenomenon known as astrogliosis (119). Astrogliosis results in the formation of scar tissue. A major function of this glial scar formation is to provide a protective barrier around damaged tissue, sealing it off from healthy tissue. Failure in axon regeneration and remyelination is often due to the presence of scar tissue. The production of many mediators by astrocytes influences T-cell responses, monocyte infiltration and microglia effector functions as well as aspects of astrogliosis and demyelination.

The mature BBB is a complex system of different cellular components. Highly specialized microvascular endothelial cells and pericytes are embedded in the basal membrane and surrounded by perivascular macrophages and astrocytic end-feet (120). Interactions with astrocytes promote strong interactions between endothelial cells resulting in a compact network sealed by tight junctions. Vascular ensheathment by astroglial processes is a unique feature of the CNS and is established at the same time as the BBB develops (121). Astrocytes and their precursors are implicated in the induction of the BBB. The BBB is the main barrier to drug transport into the brain and plays an important role in the homeostatic regulation of the brain microenvironment necessary for the stable activity of neurons.

Astrocytes produce soluble factors which help restrict development of inflammation within the CNS. Induction of expression of for example HT7, UEA-1 lectin-binding sites, and angiotensin receptors help define the phenotype of endothelial cells and induce tight junction formation (45). Astrocytes also play a key role in maintaining the ability of the CNS to limit the expansion of infiltrating T-cells by inducing apoptosis thus protecting the CNS from immune-mediated damage. T-cells that cross the BBB and invade the CNS encounter directly astrocytic end-feet that contribute to the formation of the BBB . Astrocytes express the death ligand CD95L (APO-1L, FasL) and induce apoptotic death of infiltrating T-cells by CD95/CD95L pathway (122). Defects in this mechanism of T-cell apoptosis in T-cell lines from MS patients suggest that the above mechanism of elimination is also important in preventing autoimmunity to develop (123). While astrocytes express CD95 they are not sensitive to the CD95/CD95L apoptosis pathway under normal conditions. Only at late passage (passage 8 to 10) in culture, astrocytes become sensitive to this pathway and can go into apoptosis (124). CD95 triggering in astrocytes leads to induction of CXCL8 (IL-8) which contributes to the resistance of astrocytes against CD95L. On the other hand IFN-γ results in the sensitization of astrocytes to CD95-mediated death suggesting that micro-environmental factors can influence this mechanism. Other CNS mechanisms for astrocytes to contribute to T-cell apoptosis include the release of glucocorticosteroids and NO, deprivation of IL-2 and interaction with CTLA-4 (CD152) on activated T-cells. Also, cytokines produced by astrocytes may help modulate the pro-inflammatory reactions of infiltrated T-cells (125,126).

Astrocytes also actively participate in several aspects of neuronal growth, differentiation maintenance and survival both by cell-cell interactions and by secreting neurotrophic factors. Such factors include the NGF subclass, brain-derived neurotrophic factor (BDNF) as well as neurotrophins (NT). Recently a novel neurotrophic factor has been discovered in astrocytes called mesencephalic astrocyte-derived neurotrophic factor (MANF) (127). Neuron growth and survival is also supported by astrocytic expression of growth factors and their receptors including M-CSF, GM-CSF, MCSFR and SCFR (128). Astrocytes capture and remove synaptically released glutamate (the major excitatory neurotransmitter) to end its stimulatory action and prevent neuronal damage (129). On the other hand intercellular waves of Ca^{2+} in astrocytes result in signaling to neurons as the astrocytes trigger the release of glutamate. This glutamate activates receptors on the surrounding neurons and modifies their electrical properties and synaptic functions (130). In turn, neurons influence the cellular behavior of astrocytes primarily by secretion of mediators including neurohormones and

neurotransmitters. These mediators affect several astrocyte functions such as electrophysiological responses, energy metabolism and ionic homeostasis. Rapid bidirectional neuroglial interactions are based on their specific mode of communication, synaptic transformation for neurons and gap junctional communication and Ca^{2+} waves for astrocytes (131). The impact of neurons on astrocyte differentiation seems to depend on neuronal electrical activity since it is correlated with neuronal release of GABA. Astrocytes express ion channels and receptors by which they can sense and respond to activated neurons (132). Astrocytes control neuronal growth and differentiation and guide neurons in their migration from birthplace to their final location in the CNS during brain development. Neurons and glial cells engage in a two-way dialogue from embryonic development through old age. Astrocytes influence the formation of synapses and help to determine which neural connections get stronger or weaker over time. These changes are essential to learning and to storing long-term memories (133). Furthermore, astrocytes represent a suitable substrate for *in vitro* neurite outgrowth. Neurons plated on monolayers of cultured astrocytes for example grow better, differentiate, do not aggregate and survive longer (134). Astrocytes and neurons communicate in the protection against reactive oxygen species (ROS) that are generated continuously in the human CNS during oxidative metabolism. Interactions between astrocytes and neurons and the glutathione system are involved in the defense against these ROS (135).

Microglia and the control of inflammation

Microglia are distributed throughout the CNS as a network of resting immunocompetent cells. They compose approximately 5 to 20% of all cells in the CNS and represent a distinct cell population with characteristic morphology that distinguishes them from other glial cells or neurons (136,137). Fully differentiated microglia in the mature human CNS have a highly ramified morphology. Microglia are considered as the sentinels or the gatekeepers of the brain, and they primarily appear to function as sensor cells that are continuously in surveillance of changes or insults in the CNS. Microglia provide an first line of defense against CNS-invading pathogens and their actions precede peripheral leukocyte infiltration. When activated, microglia produce a wide range of cytokines (pro- and anti-inflammatory), chemokines, NO, superoxide radicals and proteases. Microglia have the ability to present antigens to T-cells, migrate in response to chemotactic stimuli and phagocytose cell debris (138). Furthermore, microglia can induce T-cell apoptosis by direct ligation of CD95 on T-cells and they respond to signals from stressed or damaged cells (139).

As stated above, activated microglia undergo morphological and functional changes. Resting microglia in a normal CNS show extensive ramification but upon activation they loose this ramified structure and become bipolar. Upon full activation microglia transform into rounded phagocytes that can no longer be discriminated from blood-derived macrophages (140). Activated microglia proliferate and migrate through the brain. Chemokines including CCL2 (MCP-1), CCL3 (MIP-1α) and CCL4 (MIP-1β) appear to play important roles in microglia proliferation and migration (141,142,143). In parallel with the morphological changes upon activation microglia increase their production of ROS, NO, arachidonic acid derivatives as well as immunologically relevant surface markers including MHC class I and MHC class II molecules, adhesion molecules such as intercellular adhesion molecule (ICAM)-I, complement receptor 3 and other co-stimulatory molecules. Consequently, microglia become functional antigen-presenting cells (APC) (144,145,146). This is most likely relevant to MS since MHC class I and class II molecules are widely expressed by microglial cells in MS lesions (147,148,149). While it is known, however, that microglia can reactivate T-cells (150) an interesting area of new research is to define the functional consequence of this step that may very well contribute to controlling T-cell expansion rather than only promoting it. Antigen-specific interaction between APC (microglia) and T-cells is a dialogue in which both cell types regulate each other. Production of IFN-γ by T-cells is required for the maturation of resting adult microglia into APC, and for the induction of for example TNF-α production. Microglia on the other hand can not only activate T-cells, but also downregulate or modulate T-cell responses (151, 152) and they can contribute to apoptotic elimination of T-cells. For example, some microglia appear to lack the co-stimulatory molecule CD80 indicating that they are immature APC and consequently, can trigger anergy in T-cells (153) or even apoptosis (139).

Traditionally, activated microglia have been associated with tissue damage in the CNS. However, it has become increasingly clear that the role of microglia is that of a double-edged sword. Microglia may be instrumental in tissue injury through the damaging potential of their inflammatory mediators and by their phagocytic potential, but they are also beneficial in promoting homeostasis and repair. Phagocytosis by microglia for example removes undesired debris of degraded matrix and apoptotic cells and it helps to eliminate serum-derived materials from the CNS (154). In line with this function, microglia have been demonstrated to promote oligodendroglial proliferation and differentiation which contributes to myelin repair in inflammatory demyelinating disease (155) and to support axonal outgrowth

when transplanted into the spinal cord of experimental spinal cord lesions in rats (156). Also, microglia act as "stand by" glutamate scavengers in case astrocytes fail to eliminate glutamate at sufficient levels, which could contribute to neurodegeneration. Furthermore, microglia express mediators to support cell survival including neurotrophins such as BDNF (157,158, 159,160) and anti-inflammatory mediators including prostaglandin E_2 (PGE_2), TGF-β, and IL-10. These anti-inflammatory mediators inhibit both APC functions and T-cell infiltration and they counteract as production of pro-inflammatory factors by infiltrated T-cells. A marked protective role of microglia rather than only detrimental activities is also in line with the notion that brain trauma is not related to MS and certainly not a causative factor, which would be expected if microglia activation would have damaging effects only (161,162). In conclusion microglia are extremely plastic cells that exert dual functions in CNS diseases by promoting either deleterious or beneficial processes, depending on the degree, duration and context of activation.

Toll-like receptors on glial cells

Toll like receptors (TLR) were first discovered in Drosophila and later in man. TLR have been found to play a critical role in early innate immunity. So far 11 mammalian TLR have been identified that show homology to the Drosophila Toll receptor (163). Human TLR constitute a protein family linking innate and acquired immunity (164). TLR recognize conserved structural motifs expressed by microbial pathogens called pathogen-associated molecular patterns (PAMP). PAMP include various cell-wall components such as lipopolysaccharides (LPS), peptidoglycans and lipopeptides as well as flagellin, bacterial DNA and double stranded RNA. Upon activation TLR initiate a signaling cascade generally involving the proteins MyD88 and IRAK. This signaling cascade leads to the activation of transcription factors such as NF-κB that controls host defense genes including pro-inflammatory cytokines, chemokines and co-stimulatory surface molecules that not only represent key elements of innate immune responses, but also shape the ensuing adaptive immune response.

TLR are expressed in a variety of tissues, predominantly in tissues involved in immune functions such as spleen and peripheral blood leukocytes as well as in tissues exposed to the external environment including lungs and the gastrointestinal tract. However, TLR are also expressed in for example liver, bladder and kidney. Recently, their expression in the human adult CNS was documented for the first time by Bsibsi and co-workers (165). Active brain lesions from MS patients express elevated levels of at least TLR3 and TLR4

while these receptors are barely detectable in normal-appearing white matter brain sections from MS patients or in healthy white matter brain sections from controls. TLR are particularly prominent on glial cells and their expression is apparently elevated during CNS inflammation. In cell culture models human adult microglia express a wide range of different TLR, predominantly inside intracellular vesicles. Adult astrocytes in culture on the other hand predominantly express TLR4 and, to a lesser extent, TLR3. TLR are found to be expressed on the cell surface of astrocytes. TLR5 to TLR9 are not expressed at detectable levels in astrocytes, even after treatment with different stimuli. Given the dominant function of TLR in controlling innate immune responses against invading pathogens in other cell types and tissues, TLR may play a crucial role in pathogen control also in the human CNS. In this thesis we investigated the expression of TLR1, TLR2, TLR3 and TLR4 in human adult astrocytes. TLR1 and TLR2 are known to be constitutively expressed at low levels in microglia and are enhanced following *S. aureus* exposure (166). TLR2 is essential in the recognition of a variety of PAMP including bacterial lipoproteins, peptidoglycan and lipoteichoic. TLR3 is implicated in double-stranded RNA and TLR4 is predominantly activated by LPS. While TLR are now known to be expressed in human adult glial cells, the way they interact with immune regulatory functions in the CNS, however, is still unclear. TLR mediate responses to a wide range of pathogen-associated products including lipopolysaccharides, lipoproteins, flagellin, bacterial DNA and double-stranded RNA. Such responses typically involve production of a range of inflammatory mediators including TNF-α, IL-1β, IL-6, nitric oxide and type I interferons (167). We investigated expression of TLR1, TLR2, TLR3 and TLR4 in cultured human adult astrocytes after cytokine treatment as well as upon HHV-6 infection.

HHV-6, general features

HHV-6 is a double stranded DNA β-herpesvirus first isolated in 1986 as human B-lymphotropic virus (HBLV) from peripheral blood mononuclear cells (PBMC) of adults with AIDS or other lymphoprolipherative disorders (168). HHV-6 exists in two distinct variants, viz. the HHV-6A and the HHV-6B type. The overall nucleotide sequence identity between the two variants is about 90 % (169). The seropositivity for HHV-6 in the human adult population is currently estimated to be higher than 95 % (170,171). HHV-6 is generally acquired at early childhood, usually within the first two years of life (172,173,174). Children with primary HHV-6 infection obtain more often fever, diarrhea, rash and roseola as compared to children who

never acquired HHV-6 (175). Viral infection shows geographic differences with infection percentages ranging from 56 to 100 % (176,177,178,179,180, 181,182,183,184). A study in Thailand among children from 0 to 12 years indicates that the prevalence of HHV-6 infection ranges from 70 to 100 %. The highest infection percentages are found among children from 2 to 8 years and they decline in children from 8 to 12 years, similar to what has been reported for a cohort of children in Malaysia. The percentage of HHV-6 infection appears to differ somewhat in different countries: Malaysia 83 %, Brazil 77 %, Denmark 78 % (<3 years) and 100 % (>3 years), Eritrea 85 %, North China 69 %, Spain 56 %, Bratislava and New Guinea 66 %. Yet, these data are difficult to compare since the various studies pertain to different age groups. In a study of HHV-6 antibody titers rather than infection percentages (185) low titers were reported for Australia, Belgium, Israel, Japan and the USA, intermediate titers for Mexico and Germany and high titers for South Africa. In these data, no distinction could be made between the two HHV-6 variants.

HHV-6, life cycle and cell tropism

HHV-6 consists of three main structural elements: an icosahedrally symmetric nucleocapsid (90-110 nm) containing the viral DNA genome, an envelope containing the viral glycoproteins involved in receptor recognition and the tegument consisting of a protein mixture filling the space between nucleocapsid and envelope. HHV-6 is closely related to human cytomegalovirus (HCMV) (186) and enters the cell through interaction with CD46 that is present on the membrane of all nucleated cells (187). CD46 is physiologically involved in complement regulation and also serves as the receptor for measles virus (188). After binding of HHV-6 to CD46 the viral envelope fuses with the cell membrane. The incoming nucleocapsid is transported through the cytoplasm of the target cell to nuclear pore complexes and the viral DNA genome is released in the nucleoplasm. After infection of the nucleus from the target cell HHV-6 uses the cellular transcription and translation machinery to produce three kinetic classes of viral proteins: the immediate early (IE), the early (E) and the late (L) proteins. IE viral proteins are synthesized within a few hours after infection and are necessary for the production of E and L proteins. HHV-6 transmission commonly takes place through saliva and also older siblings appear to serve as a source of virus transmission (189). HHV-6 is currently known to latently infect mononuclear cells, salivary glands, lungs, genital tract, liver tissue, endothelial cells, early bone marrow progenitor cells and brain and HHV-6 proteins are present in CSF (190,191,192,193,194,195, 196,197,198,199,200). HHV-6 DNA has been detected by PCR in 32-85 %

of brain samples derived from healthy adult donors indicating that the virus is a CNS commensal (196,197,200,201,202).
HHV-6 exhibits a predominant $CD4^+$ T-cell tropism (203,204) and is rather effective in infecting a range of immortalized T-cell lines as well. The ability of the virus to infect particular T-cell lines is dependent on the variant type (205,206). Besides T-lymphocytes also fibroblasts, natural killer cells, liver cells, endothelial cells and glial cells have been successfully infected *in vitro*. Different levels of CD46 expression could possibly relate to differences in cell susceptibility to HHV-6 (207). Interestingly, infection of lymphoid tissue and $CD4^+$ T-cells with HHV-6 results in downregulation of CD46 surface expression in infected as well as in uninfected neighbour cells as assessed by staining and FACS analysis (208,209).

Clinical and immunological relevance of HHV-6
HHV-6 was first discovered in 1986 in patients with AIDS and other lymphoprolipherative disorders. HHV-6A has not been etiologically linked to any disease while HHV-6B has been implicated in clinical manifestations including exanthem subitum, fever, encephalitis, pneumonitis and hepatitis (210,211). In many disorders, the virus is suspect as a co-factor rather than a direct etiological agent. Previously in this introduction, its presumed association with MS has been discussed. In addition, anti-HHV-6 antibodies are elevated in Sjögren's syndrome, sarcoidosis, chronic fatigue syndrome as well as in AIDS patients, possibly as a secondary result of these disorders (212). In HIV infection, HHV-6 is considered as an important co-factor, most likely as the result of immunosuppressive effects of infection (see below). HHV-6 is capable of establishing latent infection in a variety of cell types. Like other latent viruses, HHV-6 can be re-activated by various conditions such as superinfection with other viruses or bacteria, immunosuppression and stress. Conversely, HHV-6 can also transactivate other viruses including HIV, HHV-7, cytomegalovirus (CMV) and EBV when these viruses are present in the same cell (213,214,215,216,217). TNF-α and IFN-γ trigger activation of latent HHV-6 in monocytes and macrophages (218,219). Interestingly, the same factors that may trigger herpesvirus reactivation, such as stress and superinfection, have also been associated with MS exacerbations (220).
A feature of HHV-6 that may be particularly relevant for its presumed role as a co-factor in several disorders is the fact that the virus codes for multiple gene products that can modulate inflammatory pathways in target cells, notably including gene products that have similarity to chemokines or their receptors (186). Amongst other functions, chemokines are involved in

directing tissue invasion by leukocytes. The U12 region of the viral genome encodes a beta chemokine receptor for CCL2, CCL3, CCL4 and CCL5 (221). The U51 region encodes a CCL5 receptor and the U83 region encodes a chemokine-like CCR2 agonist (186,222,223). Evidence suggests that these chemokine (receptor) mimics are indeed functional. U51 expression in epithelial cells results in specific binding of CCL5 and transcriptional downregulation of CCL5. U83 expressed by HHV-6-infected cells attracts cells expressing CCR2 including monocytes and macrophages and, in the CNS, astrocytes and microglia. Apart from the ability to encode chemokine (receptor) mimics, HHV-6 can influence inflammatory reactions also via CD46, its entry receptor. Recent evidence shows that for example in macrophages, HHV-6 can selectively downregulate production of the strong pro-inflammatory factor IL-12 (224), presumably by first inducing production of the anti-inflammatory mediator IL-10 (225). This effect was independent on viral replication and shown to be the direct consequence of CD46 ligation. Thus, also in this way HHV-6 may very well modulate inflammatory pathways.

Goal of the study and experimental approach

The primary goal of this study was to examine the impact of HHV-6 and EBV on inflammatory pathways in human adult glial cells. By clarifying this impact, we aimed to collect detailed information on the way these viruses may influence the development of MS. Rather than focusing on a few selected reporter genes to monitor molecular effects of viral infection, we chose a cDNA array-based gene profiling system to analyze expression levels of a wide range of 268 cytokines, chemokines, growth factors and their receptors. To perform the study, we therefore first had to examine the applicability of this comprehensive assay in terms of reproducibility and validity. Secondly, we had to optimize ways to experimentally infect cultured human glial cells with each virus. While experimental HHV-6 infection of astrocytes could be achieved at satisfactory levels, the cells were refractory to infection with EBV. The effects of HHV-6 infection on astrocytes were carefully examined by gene profiling both under normal culturing conditions and in the context of inflammatory conditions, mimicked in culture by the addition of IL-1β, TNF-α and/or IFN-γ. Also microglia could be infected with HHV-6. The gene profile of microglia was analyzed, along with the effects of pro-inflammatory conditions but the time available to the project precluded an analysis also of the effects of HHV-6 on microglia.

As indicated above, the key to our approach was the use of cultured human glial cells derived from post-mortem brain samples. In our studies, human

adult astrocytes were generally used at passage 4 and human adult microglia were used within 1 or 2 weeks after isolation. In this state, these cells are expected to mimic authentic glial cells much better than immortalized cell lines. This approach therefore appears attractive to model inflammatory pathways in the human CNS and in fact, it is the only approach to address the above research question involving a virus that only infects humans and some primates but no routine laboratory animals such as rodents. Yet, at the start of the project it was insufficiently clear to what extent cultured glial cells are representative for glial cells in an intact CNS *in vivo*. Furthermore, it was not clear whether glial cells from different donors would display similar gene profiles. These issues became part of the research question.
Glial cells used in this thesis were obtained from post-mortem sub cortical white matter kindly provided by the Netherlands brain bank (NBB) (www.hersenbank.nl). The NBB operates since 1985 and provides tissue on request, after prior submission of research protocols. The NBB supplies post mortem material from clinically well documented and neuropathologically confirmed cases and serves as a link between clinicians, neuropathologists and biomedical researchers. The NBB has three unique features. First, all donors and their next of kin are requested to sign an informed consent, when joining the program. Secondly, human brain tissue is obtained by means of rapid autopsies with a very short post-mortem delay, ranging between two to six hours minimizing post-mortem decay. Thirdly, the NBB uses a fresh brain dissection procedure, required to obtain high-quality samples for cell culture and neurochemical, immunocytochemical and metabolic studies. With the assistance of this unique facility, our studies were made possible. In present thesis we intend to clarify the impact of HHV-6 infection on glial cell functioning, neurodegeneration and demyelination.

Outline of the thesis

Following the General Introduction **Chapter 2** describes the gene expression profiles in cultured human adult astrocytes using the above-mentioned cDNA arrays. Astrocytes were chosen as a first target since they play key roles in CNS development, inflammation and repair and in regulating the blood-brain barrier. Also, it is known that HHV-6 can experimentally infect astrocytes in culture. A first important issue to address was the sensitivity, robustness and reproducibility of cDNA array-based gene profiling in cultured human adult astrocytes. Expression profiles were therefore generated eight times over for astrocyte cultures derived from a single donor to examine reproducibility, and another two cultures from different donors were examined as well. As a next step, the responses of

astrocytes to TNF-α, IL-1β and IFN-γ were monitored. This allowed comparison of the behavior of cultured astrocytes with data on astrocyte responses during inflammation *in vivo*, for example during MS, and data on similar *in vitro* models of astrocytes reported by others. **Chapter 3** describes the efforts to experimentally infect astrocytes with HHV-6, aiming at a maximum level of infection. HHV-6 is somewhat difficult to work with since it can only infect cells while "jumping" from one cell to the next. To infect astrocytes, another cell therefore had to be used as a source of virus. In **Chapter 4** experiments are described that led to documentation of the gene expression profiles of HHV-6-infected astrocytes. To examine what would happen during inflammation, infected astrocytes were next supplied with the pro-inflammatory cytokines whose effects had been studied in Chapter 2. Apart from cytokines and chemokines, also Toll-like receptor (TLR) are key players in regulating inflammatory pathways, also in the human CNS. Upon recognizing bacterial or viral structures, TLR tend to activate inflammatoryreactions. Previous research in our group had documented the presence of TLR on astrocytes and in **Chapter 5,** the possible influence of HHV-6 on these TLR was therefore explored. Astrocytes were infected with HHV-6 and expression levels of TLR1 to TLR4 were monitored by real-time quantitative PCR and compared to cytokine production that was monitored via cDNA array profiling. As a final step in the study, attention was directed at another major type of glial cell in the human CNS, microglia. Microglia are somewhat more difficult to investigate than astrocytes since they do not survive freezing and thus cannot be stored. They must therefore be studied in primary cultures, which is only possible, over a period of 2 to 3 weeks at most. Also, their yield from tissue samples varies considerably from one case to the next. **Chapter 6** describes gene profiling of purified microglia that were all cultured under standardized conditions. Presenting a general discussion, **Chapter 7** summarizes the major results of the work, evaluates the suitablity and limitations of the currently applied cDNA array gene profiling technology and attempts to put the various results into perspective.

CHAPTER 2

Cytokine, chemokine and growth factor gene profiling of cultured human astrocytes after exposure to pro-inflammatory stimuli

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Abstract
Astrocytes play key roles in CNS development, inflammation and repair by producing a wide variety of cytokines, chemokines and growth factors. Understanding the regulation of this network is important for a full understanding of astrocyte functioning. In this study, expression levels of 268 genes encoding cytokines, chemokines, growth factors and their receptors were established in cultured human adult astrocytes using cDNA arrays. Also, changes in this gene profile were determined following treatment with TNF-α, IL-1β and IFN-γ.
The data obtained reveal a highly reproducible pattern of gene expression not only between different astrocyte cultures from a single source, but also between astrocytes from different donors. Cultured human adult astrocytes were found to express receptors for TNF-α, IL-1β and IFN-γ. We also identified several gene products not previously described for human astrocytes including for example IL-17, CD70, CD147 and BIGH3. When stimulated with TNF-α, astrocytes respond with increased expression of several genes notably including those encoding the chemokines CCL2 (MCP-1), CCL5 (RANTES) and CXCL8 (IL-8), growth factors including BMP-2A, BMP-3, neuromodulin (GAP43), BDNF and G-CSF and receptors such as the CRF-receptor, the calcitonin receptor (CTR) and TKT. The response to IL-1β involves largely the same range of genes, but responses were blunted in comparison to the TNF-α response. Treatment with IFN-γ had no or only marginal effects on expression of any of the 268 genes analyzed. Astrocytes treated with a mixture of all three stimuli together displayed responses that are largely similar to those found in response to TNF-α or IL-1β alone, with only few additional synergistic effects.

Introduction

Astrocytes are the most abundant glial cell type in the human brain. They play a key role in development, homeostasis, inflammatory responses and repair in the CNS by producing a wide variety of cytokines, chemokines, growth factors and by expressing receptors for these molecules. Well-known astrocyte functions include maintenance of the blood-brain barrier (BBB), trophic support of neurons and oligodendrocytes and participation in the regulation of local inflammatory responses. Cytokines, chemokines and their receptors on astrocytes are also key in the cross talk between infiltrating cells of the immune system and the brain that determine inflammatory, neuroendocrine and even behavioral responses (1). Under inflammatory or otherwise pathological conditions astrocytes become activated and display enhanced production of several cytokines, chemokines and growth factors. Also, astrocytes generally start to proliferate under such conditions, a phenomenon known as astrogliosis (2). Under these conditions, astrocytes produce a wide variety of cytokines, chemokines and growth factors including IL-2, IL-4, IL-10, CCL2 (monocyte chemotactic protein-1), CCL5 (RANTES), CXCL8 (IL-8), TGF-β, brain-derived neurotrophic factor (BDNF) and TNF-α. Different techniques have been used to reveal this (Table2.1).

Also, data are accumulating to document the functional role of these cytokines, chemokines and growth factors in mediating astrocyte functions. For example CCL2, CCL5, CXCL8 and TNF-α are associated with recruitment of immune cells via induction of adhesion molecule expression, and with the activation of such cells, which may lead to inflammatory tissue damage as seen in multiple sclerosis (MS) (18,29). On the other hand, IL-4, IL-10 and TGF-β promote regulatory roles of astrocytes in local inflammatory response and in repair functions (38). Growth factors such as for example BDNF and its receptor (gp145trkB) are involved in immune-mediated neuroprotective interactions (39).

Table 2.1. Cytokines, chemokines, growth factors and their receptors expressed in human astrocytes

cytokine/chemokine and receptors	material	reference
control brain		
CCL2 (MCP-1)	adult	3
IL-4R	adult	4
TGF-βR1, TGF-βR2	adult	5
FGFR-1	adult	6
CXCL8 (IL-8)	adult	7
CXCR1 (IL-8R)	adult	8
CXCR3	adult	9
CXCR4	adult	7
CX3CL1, CX3CR1	adult	10
CCL2, CCR-2	fetal	11
CX3CL1 (fractalkine)	fetal	12
mesencephalic astrocyte-derived neurotrophic factor (MANF)	fetal	13
MS brain		
CCL2	adult	14
CCL3 (MIP-1α)	adult	15
CCL4 (MIP-1β)	adult	16
CCL5 (RANTES)	adult	17
CCL7 (MCP-3)	adult	14
CCL8 (MCP-2)	adult	14
IL-2	adult	18
IL-4	adult	19
IL-6	adult	20
IL-10	adult	21
TNF-α	adult	21
TGF-β	adult	21
TGF-β1, TGF-β2, TGF-β3	adult	5
TGF-βR1, TGF-βR2	adult	5
IFN-γ	adult	22
CCR3	adult	23
CCR5	adult	23
CXCL10 (IP-10)	adult	24
CXCL9 (MIG)	adult	25
IL-4R	adult	19
IL-10R	adult	19
BDNF, BDNFR	adult	26
NGFR	adult	27
CXCR3	adult	28

cytokine/chemokine and receptors	material	reference
cultured control astrocytes		
CCL2	adult	29
IL-4	adult	19
IL-10	adult	19
IL-1R2	adult	30
IL-4R	adult	4
TGF-β1, TGF-β2	adult	5
CXCR2	adult	31
CXCR4	adult	7
TNFR	adult/fetal	30
IL-1R1	adult/fetal	30
IFN-αR, IFN-βR, IFN-γR	adult/fetal	30
MCSFR, SCFR	adult/fetal	30
IL-1β,	fetal	32
TNF-α	fetal	32
IL-6	fetal	32
CXCL8	fetal	32
CXCL9	fetal	32
CXCL10	fetal	32
CXCL12	fetal	32
CCL2	fetal	32
CCL3	fetal	33
CCL4	fetal	32
CCL5	fetal	11
CCR2	fetal	34
CCR3	fetal	32
CCR5	fetal	32
CCR8	fetal	32
CXCR3	fetal	32
CXCR5	fetal	32
CXCR6	fetal	32
CX3CR1	fetal	32
BDNF	fetal	35
NGF	fetal	35
M-CSF	fetal	36
IL-6R	fetal	37

Much of what is currently known about cytokine and chemokine signaling by human astrocytes is derived from immunohistochemical studies of cytokines and chemokines in brain samples from cases of MS, Alzheimer's disease (AD) and Parkinson's disease. Also, several studies have been performed on cultured astrocytes isolated from either fetal or human adult brain tissue. In these latter types of studies, TNF-α, IL-1β and IFN-γ are frequently used as astrocyte stimulators since these cytokines represent major signaling molecules in the CNS (40). Several reports have documented the effects of these pro-inflammatory cytokines on the production of selected sets of cytokines and chemokines by astrocytes. In the discussion section of this paper, results of these studies will be revisited in the light of our own data. Despite the wealth of information on cytokine and chemokine production by astrocytes, many questions remain with regard to their function and how neurodegenerative processes and viral infection modulate this network. Experimental approaches to understand the functional roles of astrocytes may be well served by robust *in vitro* approaches that allow experimental manipulation and molecular monitoring in ways that would be impossible with intact brains or using experimental animal models. A prerequisite for the use of *in vitro* systems, however, is that astrocytes behave in a stable manner when cultured and that astrocytes from different healthy control donors behave largely similar. Gene expression profiling by cDNA arrays appears a suitable technique to evaluate this issue. Array approaches have been applied to an increasing variety of cells and tissues (41) including for example rat microglia (42), but not yet to cultured human adult astrocytes.

In this study, we first focused on the issue of robustness and reproducibility of cDNA array data. To allow more complex studies on gene expression profiles, it is necessary to establish that cultured astrocytes display a stable cytokine and chemokine gene expression profile that can serve as a reference for future studies. Rather than collecting data for thousands of genes, the cDNA array approach we have chosen was aimed at collecting reference data only for a relevant functional group of 268 genes encoding cytokines, chemokines, growth factors and their receptors, which allows for relatively easy data processing. Our results provide such a set of reference data and illustrate that cultured human adult astrocytes from different healthy control donors display a remarkably stable cytokine profile. As a next step we investigated the effects of TNF-α, IL-1β and IFN-γ on cultured human adult astrocytes.

Material and methods

Donors

Astrocytes used in the experiments were obtained from post-mortem sub cortical brain white matter provided by the Netherlands Brain Bank after rapid autopsy (post-mortem delays of less than 10 h). Three different donors were used, each of which was free from any clinical or neuropathological sign of CNS disorders. All 3 donors were female with the following characteristics: donor 1 (no 00- 022, aged 83, acute myocardial infarction), donor 2 (no 00-031, aged 77, cardiac infarction) and donor 3 (no 00-032, aged 78, decompensatio cordis).

Isolation and *in vitro* culture of human adult astrocytes

Human adult post-mortem astrocytes were obtained as previously described (43,44). Briefly, white matter samples were collected and meninges and visible blood vessels were removed before mincing the tissue into small cubes. The tissue fragments were incubated at 37°C for 20 min in 0.25 % trypsin (Sigma, St. Louis, MO) and 0.1 mg/mL bovine pancreatic DNAse I (Boehringer Mannheim, Germany). After digestion, cell suspensions were gently triturated, washed and taken into culture in medium containing 1:1 v/v DMEM : HAM-F10 supplemented with 10 % FCS (Biowhittaker, Verviers, Belgium) and pyruvate, glutamine and antibiotics. Astrocyte cultures were grown in 75 cm^2 culture flasks coated with poly-L-lysine (Sigma Chemical Co, St Louis, MO). Astrocytes were grown until post-confluent monolayers were obtained at passage 4. The purity of the astrocyte culture was verified by staining with rabbit anti-glial fibrillary acidic protein (GFAP; ZYMED, San Francisco, CA) as an astrocyte marker, murine anti-human CD68 (DAKO, Glostrup, Denmark) as a microglia marker, and murine anti-myelin basic protein (MBP; Boehringer Mannheim, Germany) as an oligodendrocyte marker. The astrocyte cultures were found to be essentially 100 % GFAP-positive. No cells were found to express CD68 or MBP.

Astrocyte treatment

Post-confluent monolayers of astrocytes (donor 00-022) at passage 4 were treated for 48 h with 500 U/mL of either recombinant human TNF-α, IL-1β or IFN-γ (PreproTech, Rocky Hill, NY), or with a mixture of each of these three cytokines at the above-mentioned concentrations.

Analysis of mRNA expression using cDNA arrays

Analysis of the mRNA profile of astrocytes was performed by hybrid selection of radioactive labeled cDNA on high-density Clontech Atlas® arrays of membrane-bound cDNA probes, according to the manufacturer's instructions and using reagents provided by the manufacturer (http://www.bdbiosciences.com/clontech). Briefly, total cellular RNA was extracted from the astrocytes using denaturing solution and treated with RNase-free DNase. Poly A^+ RNA was separated from total RNA using biotin-labeled oligo (dT) and streptavidin-labeled magnetic beads. Poly A^+ RNA was incubated with a mixture of primers for all the genes located on the array. Radioactive cDNA probes were prepared by adding dNTP-mix (for dATP label), DTT, MMLV RT and [^{32}P]-dATP and incubating for 25 minutes at 50°C. Hybridization of the radioactive cDNA probes to the membrane was carried out overnight at 68°C using continuously turning bottles. The membrane was washed in 2x saline sodium citrate (SSC), 1 % SDS and in 0.1x SSC, 0.5 % SDS at 68°C. Finally the membrane was once washed in 2x SSC at room temperature and directly packed to prevent drying. Specifically bound radioactivity was analyzed and quantitated by phosphor-imaging.

Data analysis

The hybridization signal for each gene probe, present in duplicate on the array, was calculated as the mean of these duplicates, corrected for background intensity and quantified using software provided by the manufacturer. Relative hybridization signals were calculated by dividing the absolute signals by the mean signal for all 9 housekeeping reference genes on the corresponding array, and multiplying this ratio by 1,000.

Results

Repeated cytokine and chemokine profiling for astrocytes from a single control donor and comparison to other donors

The first element in our study was to test reproducibility of the cytokine, chemokine and growth factor (receptor) gene profile in cultured astrocytes as monitored by the cDNA array. To this end, gene profiling was performed eight times, each time using a separately prepared post-confluent culture of astrocytes seeded from a single frozen stock obtained from one control donor. Also, gene profiling was performed on astrocyte cultures from two additional control donors, to examine possible donor-to-donor variation.

Overall, the gene profiles were remarkably similar in all cases. Table 2.2 lists the twenty most abundantly expressed cytokines, chemokines, growth factors and their receptors, standard deviations and coefficients of variation for gene expression signals as determined by the eight separate analyses for donor 1. Also, Table 2.2 provides a comparison of these data to those obtained with astrocytes from two additional donors. The most abundantly expressed genes include low affinity nerve growth factor receptor (NGFR), insulin-like growth factor binding protein 2 (IGFBP2), thymosin beta-10 (TMSB10), pleiotrophin (PTN), CD70 (CD27 ligand), CD147 (basigin, neurothelin), vascular endothelial growth factor receptor 1 (VEGFR1), insulin receptor (INSR) and epidermal growth factor receptor (EGFR). These primarily represent soluble mediators for proliferation and their receptors, which is not unexpected for normal cultured astrocytes.

Repeated gene profiling analysis of astrocytes from donor 1 indicated excellent reproducibility of the data, with standard deviations in the order of 5 %. This emphasizes that the gene profile of separate astrocyte cultures derived from one donor is a very stable feature. Importantly, an even better reproducibility was observed when comparing the data for astrocytes derived from donor 1 with those for two additional control donors, as evaluated by principal component analysis (PCA). This analysis indicates that the agreement between the eight data sets for donor 1 is 80 %, and the agreement between the three different donors is 93.7 %. Following transformation of the data, these figures are 90.3 % and 97.2 %, respectively (45).

Table 2.2. The twenty most abundantly expressed genes in cultured human adult astrocytes.

cytokine, chemokine or receptor	Average signal intensity[1] donor 1	SD[2] donor 1	CV (%)[3] donor 1	Signal intensity donor 2	Signal intensity donor 3	SD donor 1,2 and 3	CV	Gene code
low affinity nerve growth factor receptor (NGFR)	1232	47	3.9	1184	1194	25	2.1	M14764
insulin-like growth factor binding protein 2 (IGFBP2)	1159	48	4.1	1059	1067	56	5.1	M35410
thymosin beta-10 (TMSB10)	1148	84	7.3	1005	1116	75	6.9	M92381
pleiotrophin (PTN) + human nerve growth factor	1075	57	5.3	971	1050	54	5.3	X52946+M57399
CD70 (CD27 ligand)	1029	48	4.7	931	927	58	6.0	L08096
CD147 (basigin, neurothelin)	1005	77	7.6	923	988	43	4.5	L20471
vascular endothelial growth factor receptor 1 (VEGFR1)	970	47	4.9	871	888	53	5.8	X51602
insulin receptor (INSR)	864	59	6.8	773	889	61	7.3	M10051
epidermal growth factor receptor (EGFR)	863	56	6.5	789	903	58	6.8	X00588
interleukin-6 (IL-6)	806	318	39.4	637	609	107	15.6	X04602
BIGH3	792	79	10.0	785	622	96	13.1	M77349
CCL2 (monocyte chemotactic protein 1, MCP-1)	778	122	15.7	679	884	103	13.1	M24545
interleukin-4 (IL-4)	770	230	29.8	637	677	68	9.8	M13982
vascular endothelial growth factor (VEGF)	766	49	6.4	699	802	52	6.9	M32977
fibroblast growth factor receptor 1 (FGFR1)	764	38	5.0	677	775	54	7.3	M34641
UFO/Axl receptor tyrosine kinase	762	66	8.7	759	692	40	5.4	M76125
TEK/TIE-2 receptor tyrosine kinase	717	46	6.4	667	700	25	3.7	L06139
tumor necrosis factor alpha (TNF-α)	703	73	10.4	700	742	23	3.3	X01394
connective tissue growth factor (CTGF)	702	45	6.3	732	745	22	3.0	M92934
bone morphogenetic protein 2A (BMP-2A)	695	228	32.9	533	586	83	13.7	M22489

[1] The average for donor 1 is based on eight separate analyses , [2] standard deviation , [3] coefficient of variation

Signals for a minority of genes including CCL2, IL-4, IL-6, TNF-α and BMP-2A show a somewhat higher standard deviation, varying from 10 % up to as much as 40 %. Since the vast majority of genes on the arrays produce a highly reproducible expression signal, the varying signals for these few genes most likely indicate intrinsic variations in the expression levels of these particular genes in the astrocyte cultures. Interestingly, CCL2, IL-4, IL-6, TNF-α and BMP-2A are known to be highly inducible in human astrocytes (see below and Tables 2.3 and 2.4) (46,47). Therefore, it seems that these particular inducible genes respond to subtle variations in cell culturing conditions and astrocyte densities at the time of RNA harvesting.

Cytokine and chemokine profiling of astrocytes treated with TNF-α, IL1-β, IFN-γ, or a mixture of these three cytokines together

Having established reproducibility of the gene profile in cultured astrocytes, the effect of pro-inflammatory stimuli including TNF-α, IL1β and IFN-γ was examined next. The astrocytes were found to express the receptors for these three cytokines. The concentrations of the cytokines used for treatment as well as the time for treatment were chosen on the basis of our previous and current gene expression studies using cultured human adult astrocytes (43). Under the conditions used by us, the most pronounced changes in mRNA levels for a variety of cytokine and stress protein genes in astrocytes occur over periods of 6 to 72 h. In line with our findings, Croitoru-Lamoury and co-workers recently reported that for several chemokines in human fetal astrocytes, gene induction effects by TNF-α, IL-1β and IFN-γ generally accumulated over 72-h periods (32). Based on this, we considered 48 h as a suitable time point allowing us to monitor relevant changes for most genes. Gene profiling of IFN-γ treated astrocytes was also performed after 6 or 24 h to verify that no relevant responses escaped detection by focusing only on the results obtained after 48 h (see below).

Genes induced by TNF-α

Treatment of astrocytes with TNF-α resulted in markedly elevated expression of a distinct group of genes fulfilling different functions. No genes were found whose expression was markedly decreased as the result of treatment.

Figure 2.1 depicts a complete overview of the relative levels of expression of all genes on the array following treatment as compared to their expression in untreated astrocytes. Several genes can be found in the upper left part of Figure 2.1, representing genes whose expression is enhanced by TNF-α.

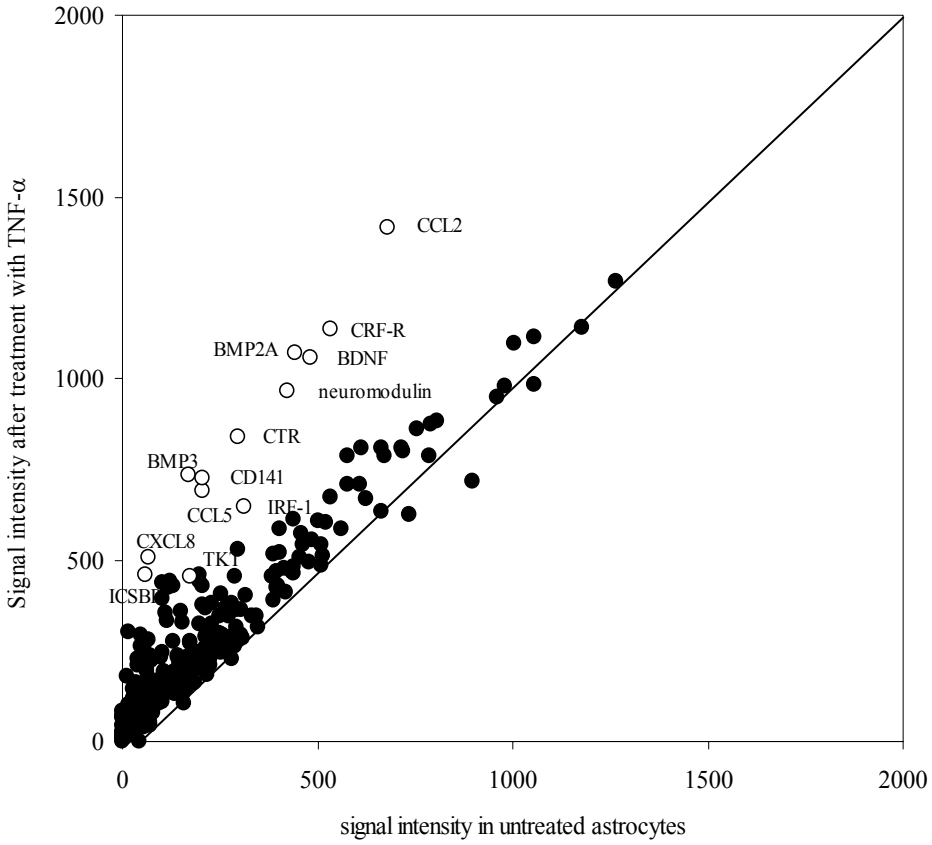

Figure 2.1. The impact of TNF-α on human astrocyte genes encoding cytokines, chemokines, growth factors and their receptors
Cultured human adult astrocytes were treated with TNF-α for 48 h and using a cDNA array, expression levels of 268 cytokines, chemokines, growth factors and their receptors were determined and compared to levels found in untreated astrocytes. Signals at or around the diagonal line represent genes whose expression is unaffected by TNF-α treatment. Signals in the upper left part of Figure 2.1 represent genes whose expression is enhanced by the treatment. The data represent the averaged result of two separate experiments. For further details, see materials and methods.

Table 2.3 shows a summary of genes whose exression was elevated by TNF-α (or any of the other stimuli in the present study). As major genes, we considered genes with relative signals of at least 300 in untreated astrocytes, reflecting a level of expression that is at least 30 % of that of the average for all housekeeping genes analyzed as reference. Changes in expression levels for these genes are considered meaningful when they exceeded a factor of 2. For minor genes of interest, with a minimal relative signal of 250 in treated astrocytes, changes in expression levels were considered meaningful when they exceeded a factor of 3.

The most strongly induced major genes after TNF-α treatment include CCL2 (MCP-1, a strong chemotactic factor involved for example in leukocyte recruitment across the BBB), BMP-2A and BMP-3 (mediators in neural development), corticotrophin-releasing factor receptor 1 (CRF-R, a neuromodulatory hormone receptor), BDNF (a neurotrophic factor), neuromodulin (GAP43, a neuroprotective factor), interferon regulatory factor-1 (IRF-1, a transcription factor regulating interferon responses), calcitonin receptor (CTR), CCL5 (RANTES, a chemotactic factor) and receptor protein tyrosine kinase (TKT).

Following treatment with TNF-α, several minor genes are induced including CD141 (a receptor normally associated with thrombin binding and coagulation), cytokine receptor EBI3, tumor necrosis factor receptor 1 (TNFR1) and TNFR2, interleukin-9 receptor (IL-9R), transforming growth factor-alpha (TGF-α), IL-17, CXCL6 (GCP 2), CXCL8 (IL-8), fibroblast growth factor 7 (FGF7), CXCL9 (interferon-gamma-induced monokine, MIG), CD114 (the G-CSF receptor), CCL4 (MIP-1β), interferon consensus binding sequence-protein (ICSBP), and granulocyte colony-stimulating factor (G-CSF).

Genes induced by IL-1β

Treatment of astrocytes with IL1-β resulted in effects on gene expression that are clearly reminiscent of the effects of TNF-α, but that are less pronounced. Figure 2.2 depicts a complete overview of the relative levels of expression of all genes on the array following treatment as compared to their expression in untreated astrocytes. No genes were found whose expression was markedly decreased as the result of treatment. Table 2.3 highlights the most important effects of IL-1β treatment. Concerning the major astrocyte genes, only the receptor protein tyrosine kinase (TKT) was induced by IL-1β with more than a factor of 2. For the minor genes, induction was observed of erythroid differentiation protein (EDF), IL-17, CD135 (STK1), CXCL8 (IL-8), vitamin B3 receptor (G-protein-coupled

receptor HM74), FGF7 and granulocyte colony-stimulating factor (G-CSF). In addition, several other genes displayed modestly enhanced levels of expression (yet below the arbitrary threshold of a factor of 2). This group of genes is virtually identical to the group of genes induced by TNF-α, including again for example CCL2, BMP-2A, BMP3, CRF-R, BDNF and CTR.

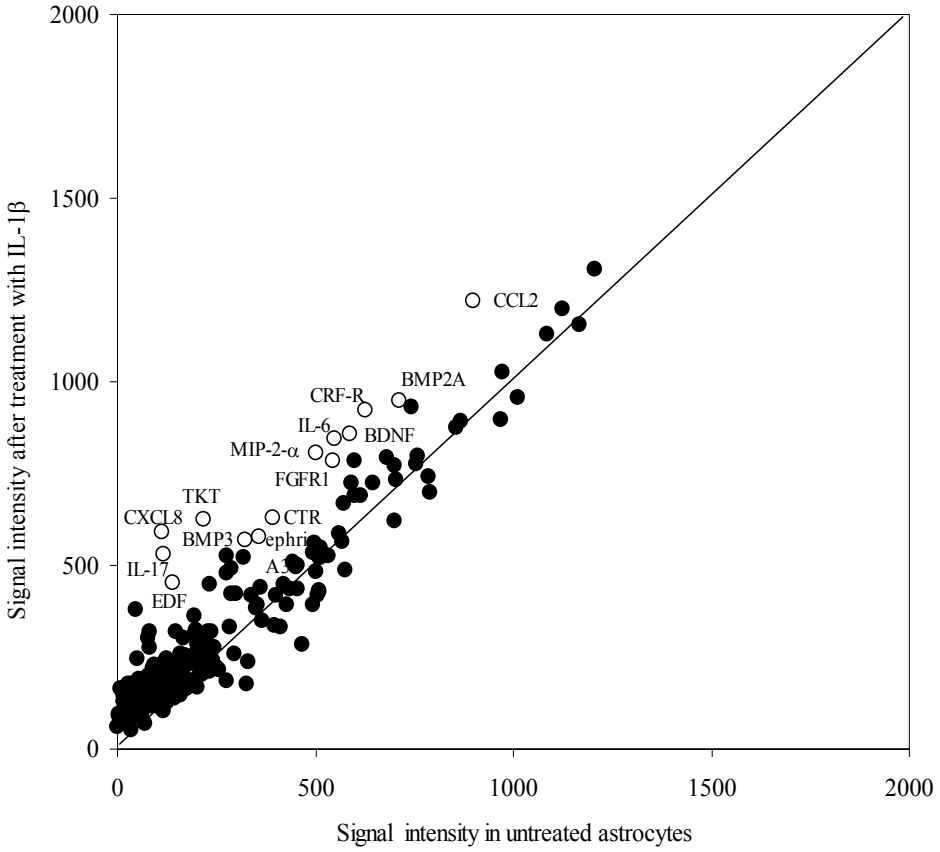

Figure 2.2. The impact of IL-1β on human astrocyte genes encoding cytokines, chemokines, growth factors and their receptors
Cultured human adult astrocytes were treated with IL-1β for 48 h and using a cDNA array, expression levels of 268 cytokines, chemokines, growth factors and their receptors were determined and compared to levels found in untreated astrocytes. Signals at or around the diagonal line represent genes whose expression is unaffected by IL-1β treatment. Signals in the upper left part of Figure 2.2 represent genes whose expression is enhanced by the treatment. The data represent the averaged result of two separate experiments. For further details, see materials and methods.

The effects of IFN-γ

In contrast to the effects induced by either TNF-α or IL-1β, treatment of astrocytes with IFN-γ had no marked effect on levels of expression of any of the genes encoding cytokines, chemokines, growth factors and their receptors analyzed in the present study (Figure 2.3 and Table 2.3). In order to examine whether the 48-h treatment time would perhaps obscure more rapid effects on gene expression, the analysis was repeated with astrocytes treated with IFN-γ for only 6 or 24 h. After a 6-h treatment, only the abundantly expressed CCL2 (MCP-1) showed a relative increase of more than a factor 2, *viz*. 3.35 (data not shown). After 24 h treatment, only glial growth factor and BDNF were induced by a factor of 3.71 and 3.40, respectively. This result indicated that marked induction of notably CCL2, glial growth factor and BDNF by IFN-γ does occur but that this is a relatively rapid response only detectable after 6 or 24 h. No other major gene appears to be induced by IFN-γ after 6 or 24 h of treatment any more than after 48 h.

The effects of a mixture of TNF-α, IL-1β, and IFN-γ

Treatment of astrocytes with a mixture of the cytokines TNF-α, IL-1β, and IFN-γ resulted in distinct upregulation of several genes. Again, no genes were found whose expression was downregulated by the treatment. Figure 2.4 shows the expression signals for treated astrocytes versus untreated ones, revealing a group of genes upregulated by the mixture of cytokines that is largely the same as the group induced by the individual cytokines TNF-α or IL-1β. Table 2.3 highlights the effects of the combined treatment with TNF-α, IL-1β, and IFN-γ. Major genes upregulated by a factor 2 or more include for example CRF-R, BDNF, neuromodulin, CTR, BMP-3 and TKT that were all already found to be upregulated by either TNF-α or IL-1β alone. CCL2 was, on the other hand, upregulated by a factor of only 1.75. Following treatment with TNF-α, IL-1β, and IFN-γ several minor genes are upregulated including IL-11, IL-17, CXCL8 (IL-8), CD114 (the G-CSF receptor), granulocyte colony-stimulating factor (G-CSF), insulin-like growth factor II (IGF2), and erythropoietin receptor (EPOR).

Previous reports have indicated the possibility of synergistic effects of cytokines on astrocyte gene expression. Of all 268 genes, only IGFBP3, CXCL2 (MIP2-α), ephrin A3, follistatin-related protein, neurotrophin-3, IL-11, IGF-2 and erythropoietin receptor were found to be markedly induced by the mixture of cytokines while being less affected by each of the cytokines individually. Overall, however, synergistic effects between TNF-

α, IL-1β and IFN-γ appear to be limited since the group of genes that is induced by the mixture of these mediators primarily reflects additive effects of TNF-α and IL-1β alone.

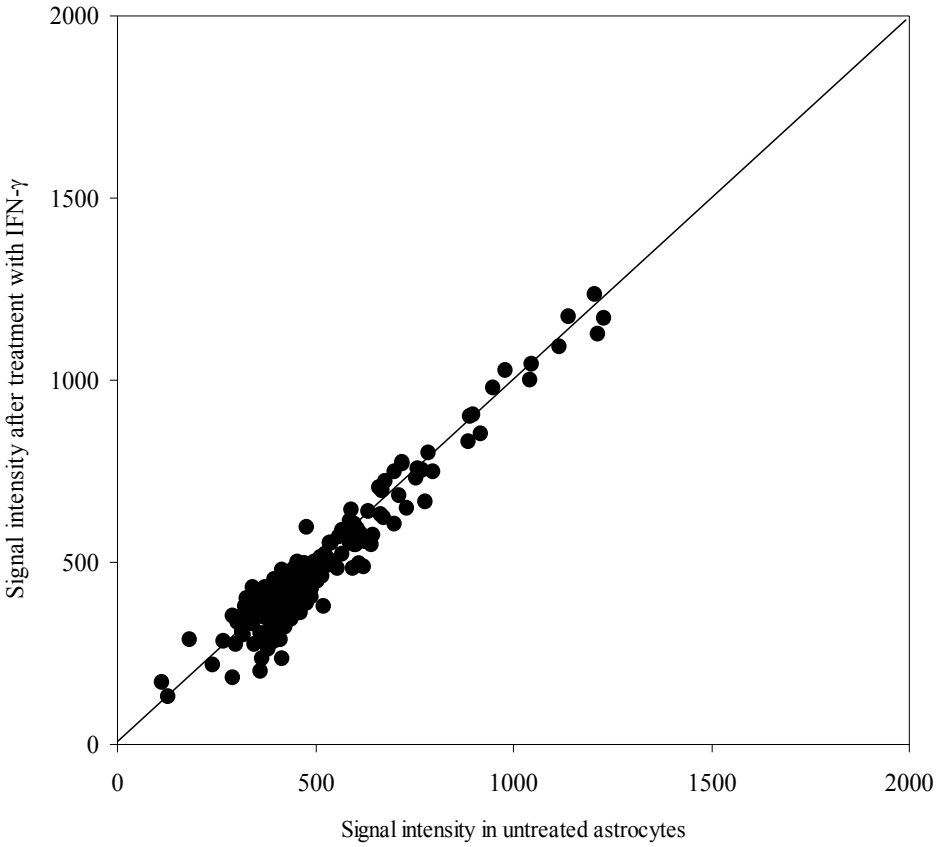

Figure 2.3. The impact of IFN-γ on human astrocyte genes encoding cytokines, chemokines, growth factors and their receptors
Cultured human adult astrocytes were treated with IFN-γ for 48 h and using a cDNA array, expression levels of 268 cytokines, chemokines, growth factors and their receptors were determined and compared to levels found in untreated astrocytes. All signals can be found in Figure 3 at or around the diagonal line indicating the absence of any gene on the array whose expression is markedly affected by IFN-γ. The data represent the averaged result of two separate experiments. For further details, see materials and methods.

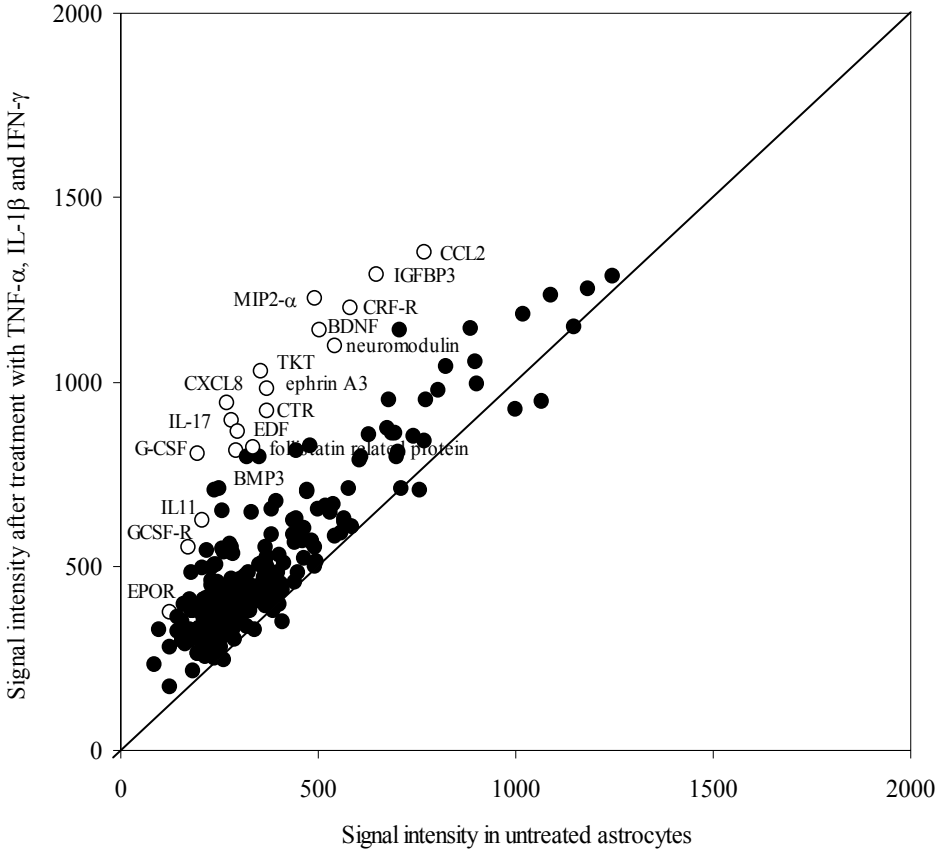

Figure 2.4. The impact of a combined treatment with TNF-α, IL-1β and IFN-γ on human astrocyte genes encoding cytokines, chemokines, growth factors and their receptors

Cultured human adult astrocytes were treated with a mixture of TNF-α, IL-1β and IFN-γ for 48 h and using a cDNA array, expression levels of 268 cytokines, chemokines, growth factors and their receptors were determined and compared to levels found in untreated astrocytes. Signals at or around the diagonal line represent genes whose expression is unaffected by the treatment. Signals in the upper left part of Figure 2.4 represent genes whose expression is enhanced. The data represent the averaged result of two separate experiments. For further details, see materials and methods.

Table 2.3. Gene profiles of human adult astrocytes after treatment with different pro-inflammatory cytokines

	treatment with TNF-α	treatment with IL-1β	treatment with IFN-γ	treatment with TNF-α, IL-1β and IFN-γ	Gene code
Major genes					
CCL2 (monocyte chemotactic protein 1, MCP-1)	**2.08**	1.36	1.00	1.75	M24545
bone morphogenetic protein 2A (BMP2A)	**2.42**	1.34	0.93	1.61	M22489
insulin-like growth factor binding protein 3 (IGFBP3)	1.26	1.17	0.88	**2.25**	M31159
corticotrophin releasing factor receptor 1 (CRF-R)	**2.13**	1.47	1.01	**2.06**	X72304
brain-derived neurotrophic factor (BDNF)	**2.18**	1.46	0.99	**2.27**	M61176
Neuromodulin (GAP43)	**2.27**	1.44	1.03	**2.02**	M25667
CXCL2 (MIP2-alpha)	1.40	1.61	1.03	**2.49**	X53799
interferon regulatory factor 1 (IRF1)	**2.07**	1.23	1.01	1.49	X14454
ephrin A3 (EFNA3)	1.33	1.76	0.92	**2.64**	U14187
calcitonin receptor (CTR)	**2.82**	1.61	1.02	**2.48**	L00587
CCL5 (RANTES)	**3.34**	1.31	0.99	1.70	M21121
follistatin-related protein	1.57	1.93	0.94	**2.43**	U06863
bone morphogenetic protein 3 (BMP3)	**4.33**	1.62	0.91	**2.77**	M22491
receptor protein tyrosine kinase (TKT)	**2.62**	**2.86**	0.88	**2.89**	X74764
neurotrophin-3 (NT-3), NGF-2	1.72	1.73	1.01	**2.25**	X53655
Minor genes					
CD141 (thrombomodulin)	**3.54**	1.89	1.05	2.49	M16552
erythroid differentiation protein (EDF)	2.41	**3.20**	0.93	2.91	J03634
interleukin-11 (IL-11)	1.06	1.41	0.57	**3.02**	M57765
cytokine receptor EBI3	**3.19**	1.85	1.00	2.85	L08187
tumor necrosis factor receptor (TNFR) + TNFR2	**4.28**	2.09	0.98	1.52	M32315 + M55994
interleukin-9 receptor (IL-9R)	**3.62**	1.64	0.99	2.01	M84747
transforming growth factor-alpha (TGF-alpha)	**3.60**	1.57	0.91	2.13	K03222
interleukin-17 (IL-17)	**3.35**	**4.53**	0.96	**3.17**	U32659
CD135 (stem cell tyrosine kinase 1, STK1)	3.03	**3.92**	0.88	2.53	U02687
CXCL6 (granulocyte chemotactic protein 2, GCP 2)	**3.80**	2.28	0.97	1.87	X78686
CXCL8 (IL-8)	**7.64**	**5.30**	0.98	**3.52**	Y00787
vitamin B3 receptor (G-protein-coupled receptor HM74)	15.23	**3.95**	0.87	2.98	D10923
fibroblast growth factor 7 (FGF7)	**5.76**	**3.44**	0.99	1.87	M60828
CXCL9 (interferon-gamma-induced monokine, MIG)	**4.50**	2.62	1.11	1.84	X72755
CD114 (GCSF-R)	**4.35**	1.99	0.94	**3.21**	M59818
CCL4 (MIP-1beta)	**6.10**	5.00	0.69	1.33	J04130
interferon consensus binding sequence-protein	**7.53**	2.22	1.11	2.16	M91196
granulocyte colony-stimulating factor (G-CSF)	**17.39**	**8.90**	0.92	**4.11**	X03438
insulin-like growth factor II (IGF2)	1.26	1.75	0.63	**3.34**	M29645
erythropoietin receptor (EPOR)	2.26	3.75	1.47	**3.03**	M60459

Numerical values represent the ratio of expression in treated *versus* untreated astrocytes. Indicated in bold are major genes (relative signals in untreated astrocytes >300) with a ratio of at least 2, and minor genes (relative signals in untreated astrocytes <300 and relative signals in treated astrocytes of over 250) with a ratio of at least 3.

Discussion

In this study, cDNA arrays were used to evaluate the expression profile in cultured human astrocytes for 268 genes encoding cytokine, chemokines, growth factors and their receptors, and the effects of TNF-α, IL-1β and IFN-γ on this profile. Gene profiles established for repeated astrocyte cultured derived from a single donor as well as for astrocytes from different donors are remarkably stable. The vast majority of gene products that are relatively abundantly expressed in untreated cultured astrocytes appear to be associated with normal regulation of proliferation and either represents soluble mediators of growth, or their receptors. Several of these products have also been found *in vivo* in reactive and proliferating astrocytes in human brain. This applies to nerve growth factor receptor (NGFR), pleiotrophin (PTN), epidermal growth factor receptor (EGFR), IL-6 and connective tissue growth factor (CTGF) (20,21,27,48,49). Our study also identifies several genes that to the best of our knowledge have not previously been documented to be expressed in astrocytes. These novel astrocyte gene products include IL-17, CD70, CD147, BIGH3, UFO/Axl receptor tyrosine kinase and TEK/TIE-2 receptor tyrosine kinase.

Treatment of astrocytes with either TNF-α or IL-1β alone leads to induction of a remarkably similar set of astrocyte genes including chemotactic factors amongst which the predominant ones are CCL2 (MCP-1), CXCL2 (MIP-2α), CXCL8 (IL-8), CCL5 (RANTES), neuroprotective factors primarily including BDNF, neuromodulin (GAP43) (50), BMP-2A and BMP-3, and receptors notably including the CRF and calcitonin receptors and receptor protein tyrosine kinase (TKT). Somewhat to our surprise, IFN-γ treatment under the conditions used by us has no appreciable effect on the level of expression of any of the 268 genes examined. We examined the possibility that a 48-h treatment time could obscure more rapid effects on gene expression. Apart from CCL2, glial growth factor, and BDNF whose expression was indeed elevated after 6 or 24 h but not after 48 h, none of the other genes were affected by IFN-γ at these earlier time points either. The effects of IFN-γ were monitored using two different fresh batches of the cytokine, ruling out the possibility that the lack of any major effect could be caused by the state of our IFN-γ preparation. IFN-γ therefore, appears to simply have very little effect on the cytokine/chemokine network in cultured human adult astrocytes, despite the fact that the IFN-γ receptor is found, by us as well as by others, to be expressed by these cells (30). Our finding is in line with several other studies that have documented the failure of IFN-γ to induce major astrocytic cytokine and chemokine mediators including CCL2,

CCL5, CXCL8, BDNF, IL-1β, IL-1α, CCL3 (MIP-1α) and CCL4 (MIP−1β) in cultured human adult astrocytes (29,33,36,48,51). Many of the previously reported effects of IFN-γ on human astrocytes (apart from its effect on IL-6, TNF-α and CXCL10) were obtained using fetal rather than adult astrocytes (Table 2.4). In particular for IFN-γ this difference has previously been shown to be of relevance since several of the stimulatory effects on fetal astrocytes did not occur when adult astrocytes were used (52,53). Lafortune and co-workers reported an increased number of cultured human adult astrocytes with detectable expression of IL-6 and TNF-α in response to IFN-γ (53), but levels of expression were not quantified in that study, making a comparison to our present data difficult. Our data leave open the possibility that IFN-γ induces CXCL10 (IP-10), as has been reported by others (29) since the cDNA arrays used in the present study do not contain a CXCL10 probe.

The mixture of all three pro-inflammatory cytokines together induced gene expression changes that were very similar to those found in response to either TNF-α or IL-1β alone. While examples of synergistic effects by cytokines on astrocytes have been reported, our results indicate that overall, such synergistic effects are limited and the result of the mixture of cytokines is largely a simple addition of the effects of the individual cytokines. The only genes with appreciable induction by the mixture of cytokines, while being much less affected by individual cytokine stimuli include IGFBP-3, CXCL2, follistatin-related protein, ephrin A3 (EFNA3), IL-11 and IGF-2.

While the technique of cDNA array gene profiling has now been well accepted, and has been found to be at least as reliable as other approaches to quantify gene expression, it is obviously of relevance to compare our data on cytokine responses by human astrocytes to those already reported by others, as a validation of the current data. In comparing our data to previously published results, it must be kept in mind that treatment times, cytokine dosing and, most importantly, the source of astrocytes are important variables that can confound direct comparison. In Table 2.4, a comparison is presented between our data and previously reported stimulatory effects of the cytokines TNF-α, IL-1β, and IFN-γ on cytokine and chemokine gene expression in cultured human astrocytes.

Table 2.4. Comparison of previously reported findings of gene induction in astrocytes with the present data

	Material	Reference	Ratio[1]
TNF-α treatment			
CXCL8 (IL-8)	adult, fetal	29, 36	7.64
CCL2 (MCP-1)	adult	29	2.08
CCL5 (RANTES)	adult	29	3.34
CCL2	fetal	55	2.08
CCL3 (MIP-1α)	fetal	56	2.25
CCL4 (MIP-1β)	fetal	56	6.10
CCL5	fetal	57	3.34
M-CSF (=CSF-1)	fetal	36	1.53
G-CSF, GM-CSF	fetal	36	17.39, 2.34
IL-6	fetal	36	1.37
IL-15	fetal	58	2.80
CCR2	fetal	32	2.09
platelet-derived growth factor (PDGF)	fetal	59	1.23
CXCL10 (IP-10)	adult	29	not on array
CX3CR1	fetal	32	not on array
IL-1β treatment			
CXCL8	adult, fetal	29,36	5.30
CXCL1	adult	60	not on array
CCL2	adult	29	1.36
CCL5	adult, fetal	29, 61	1.31
CCL3	fetal	56	7.27
CCL4	fetal	56	5.00
CCL5	fetal	57	1.31
M-CSF	fetal	36	2.50
G-CSF, GM-CSF	fetal	36	8.90, 1.48
TNF-α	fetal	54	1.04
IFN-β	fetal	61	2.52
IL-6	fetal	36	1.54
IL-15	fetal	58	1.35
IRF7	fetal	61	not on array
CXCL10 (IP-10)	adult, fetal	29,61	not on array
IFN-γ treatment			
IL-6	adult	53	0.93
TNF-α	adult	53	0.97
CCL2, CCL3	fetal	62	1.00/0.69
CCL4, CCL5	fetal	62	0.69/0.99
interferon regulatory factor-1 (IRF-1)	fetal	48	1.01
TNFαR1	fetal	63	0.93
IL-15	fetal	58	0.86
ICAM-1	fetal	48	not on array
CXCL10	adult, fetal	60, 29	not on array
TNF-α and IL-1β treatment			
CCL5	fetal	64	1.70[2]
CCL2	fetal	32	1.75[2]
TNF-α and IFN-γ treatment			
CCL5	fetal	64	1.70[2]
CCR5	fetal	32	not on array
CXCL8, CXCL9 (MIG)	fetal	32	3.52[2]/1.84[2]
CXCL10	fetal	32	not on array
CXCR4	fetal	32	not on array
CX3CL1 (fractalkine)	fetal	65	not on array
IFN-γ and IL-1β treatment			
CCL2	fetal	29	1.75[2]
CCL5	fetal	64	1.70[2]

[1] Based on data from the present study, [2] Ratio following treatment with TNF-α, IL-1β and IFN-γ

Table 2.4 illustrates that almost all cytokines and chemokines previously reported by others to be induced by either TNF-α or IL-1β alone, or by a mixture of these cytokines, were also found to be upregulated in the present study. All TNF-α inducible genes previously documented by others were reproduced by the present study, with relative increases of expression ranging from 1.37 to 17.39. As the only exception, induction of TNF-α by IL-1β as reported by Lee and co-workers (54) could not be reproduced in the present study. However, this induction was only observed after 4 to 16 h of treatment, and not after 72 h. The apparent discrepancy might therefore well be explained by different treatment times. Overall, the correspondence between the current data and previously reported effects is very good, validating our present data set.

One of the most abundantly expressed genes in astrocyte cultures, and one that is also markedly induced by both TNF-α and IL-1β is CCL2 (MCP-1). Also others have already documented the importance of CCL2 for astrocyte functions by showing its induction in cultured human astrocytes after treatment with TNF-α (66) and its elevated levels of expression under a variety of inflammatory or traumatic conditions *in vivo* in the CNS (16,67,68). CCL2 is known to regulate leukocyte transmigration across the BBB (69) and it is a prime mediator of astrogliosis (2). Another major chemotactic factor that emerges from the present study as well as from previous reports as an important inducible astrocyte gene product is CXCL8 (IL-8). CXCL8 is involved in the activation and chemotaxis of polymorphonuclear leukocytes (70) and its induction in adult as well as in fetal astrocytes by TNF-α and IL-1β is well documented (29,71).

It is also interesting to note that treatment with either TNF-α or IL-1β alone, as well as treatment with the mixture of cytokines (Table 2.3) consistently leads to a 3 - to 4.5-fold induction of IL-17, a pro-inflammatory cytokine that has so far been considered as T-cell specific (72). Our data suggest a role of IL-17 also in astrocyte functions and thus, in CNS inflammation. Interestingly, it was recently reported that IL-17 is strongly upregulated in tissue samples of chronic inactive MS plaques, but not in early active lesions (73). When combined with our data, this suggests that astrocytes are in fact responsible for marked IL-17 production at these late stages of MS plaques. As a final note, it should be emphasized that cultured astrocytes as used in the present study represents a simplification relative to the state of astrocytes in an intact CNS, where additional interactions with other cell types and matrix components are likely to influence the cytokine/chemokine network. Yet, cultured human astrocytes present a unique system to examine in detail the response of astrocytes to defined stimuli at the level of

the genome or proteome. It is our conviction that this will help further understand the complex molecular interactions that ultimately govern development, inflammation and repair in the CNS.

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CHAPTER 3

Infection of cultured human adult astrocytes with human herpesvirus-6

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Abstract
Human herpesvirus-6 (HHV-6) is a ubiquitous virus that infects about 90 % of the human population and frequently establishes latent infection in astrocytes, oligodendrocytes and microglia in the central nervous system (CNS). Since the HHV-6 genome encodes several molecules that can act as chemokine receptors or as chemokine mimics, the virus may well impact on immune regulatory processes in the CNS, such as those relevant to inflammatory neurodegenerative disorders in which glial cells play key roles.
To examine the impact of HHV-6 on glial cell functions *in vitro* models of experimental infection appear to be an attractive approach. In the present study we therefore investigated the efficiency of experimental infection of cultured human adult astrocytes with HHV-6-infected T-cell lines as a source of cell-borne virus, as well as with cell-free preparations. While cell-free preparations of HHV-6 led to infection of only about 10 % of cultured astrocytes, cell-borne virus could be reproducibly transferred to about 30-35 % of astrocytes. This level of infection could be obtained with HSB-2 T-cells but not with JJHan T-cells as carriers. Infection with HHV-6 was apparent from cytopathic effects (CPE) including syncytium formation and specific staining for HHV-6 antigen. After 3-5 days following infection, death of infected cells started to become apparent. For the first 3 days, therefore, HHV-6-infected astrocyte cultures provide a useful *in vitro* model to explore the effects of the virus on inflammatory pathways in human astrocytes.

Introduction

Human herpesvirus 6 (HHV-6) is a β-herpesvirus, first discovered in 1989 as a human B-lymphotropic virus (1) of which two variants are known, viz. HHV-6A and HHV-6B (2). Infection with HHV-6 is common in infancy and early childhood (3) and the virus persistently infects about 90 % of the human population (4,5,6). Following transmission, usually through saliva (7), HHV-6 can spread to mononuclear cells, salivary glands, lungs, genital tract and the central nervous system (CNS) via its receptor CD46 (8). In particular in the CNS, HHV-6 successfully persists and viral DNA has been detected in as many as 85 % of brain samples derived from healthy adult donors (9,10,11,12,13). Especially glial cells including astrocytes, oligodendrocytes and microglia appear to form life-long reservoirs of virus.

To date, the role of HHV-6 in human disease remains to be fully clarified. To understand the role of the virus in human disease it is therefore particularly important to dissect its impact on immune mediated processes. While the virus triggers a predominantly pro-inflammatory reaction in immortalized T-cell lines that are routinely used to propagate the virus (14), the effects in other target cells may be different. In particular in adult CNS glial cells, where infection is usually not accompanied by pathogenic immune responses, the way HHV-6 affects cell functioning remains to be established. *In vitro* approaches to this issue appear to be attractive. Successful infection of cultured human fetal astrocytes and of oligodendrocytes and microglia have been reported (15,16,17). In fetal astrocytes, infection led to rapid development of CPE and after 1 week to total lysis of the culture. In adult oligodendrocytes and microglia on the other hand, cell lysis and release of virus was very limited suggesting that adult glial cells may respond differently to HHV-6 infection from fetal glial cells.

In this study, we examined HHV-6 infection of cultured human adult astrocytes to evaluate whether such a model would allow for further studies on immune regulatory aspects of HHV-6 infection in the CNS. The goal was therefore to obtain a reproducible level of infection without cell lysis that would allow for further experimental manipulation of infected cells. We focused on HHV-6A since this variant is more frequently detected in human adult CSF and brain samples as compared to HHV-6B (9,18).

In contrast to fetal human astrocytes, HHV-6-infected adult astrocytes in culture did not undergo massive lysis but in the first three days after experimental infection, were engaged in syncytium formation only. Transmission of HHV-6 from infected to uninfected astrocytes appeared to be much less efficient than transmission from infected T-cell lines, limiting

viral spread in the culture after an initial dose of T-cell-borne HHV-6. Thus, adult astrocyte cultures could be established in which about one third of the cells were stably infected as assessed by cytopathic effects (CPE) and staining for specific HHV-6 antigen. Such infected cultures may provide a useful *in vitro* research model to explore the effects of HHV-6 on inflammatory pathways in the human CNS.

Materials and methods

HHV-6 infection of human T-cell lines

HHV-6A GS and U1102 were grown using the HSB-2 and the JJHan T-cell lines (19,20,21). HHV-6-infected HSB-2 T-cells were prepared by co-culturing 5.5 x 10^6 uninfected cells/mL with one tenth of total cell volume HHV-6-infected HSB-2 T-cells (GS strain; multiplicity of infection (MOI) 0.1) in RPMI 1640 (Biowhittaker, Verviers, Belgium) supplemented with 10 % FCS (Biowhittaker, Verviers, Belgium) and antibiotics at 37ºC in a humidified atmosphere containing 5 % CO_2. After 2 h at 37ºC infected T-cells were collected by centrifugation at 210 g for 10 min, resuspended in culture medium and cultured in 75 cm^2 flasks kept upright (25 mL/flask). After 2 days in culture levels of 40 % infection were reached and cultures were split into two equal parts. After an additional culture period of 3 days 75 % of the HSB-2 T-cells were infected. Infected HSB-2 T-cells from the separate cultures were collected centrifuged at 210 g for 10 min and stored at 0.5 x 10^6 cells/mL in 90 % v/v FCS and 10 % v/v dimethylsulfoxide (DMSO; Merck, Darmstadt, Germany) at -150ºC.

HHV-6-infected JJHan T-cells were prepared by infecting 4 x 10^6 non-infected cells with HHV-6-infected cells (U1102 strain, tissue culture infectious dose 50 ($TCID_{50}$) = 1 x 10^6) (19); supplied by U. Gompels, London School of Hygiene and Tropical Medicine, London. After 4 days in culture levels of 60 % infection were reached. HHV-6-infected T-cells were centrifuged at 210 g for 10 min and stored at 0.5 x 10^6 cells/mL in 90 % v/v FCS and 10 % v/v dimethylsulfoxide at -150ºC.

To determine the levels of infection in cell cultures, as defined by the percentage of infected cells rather than by virus titers, samples of infected cultures were fixed in acetone supplemented with 0.03 % v/v H_2O_2 for 10 min. Cells were incubated overnight at 4ºC in PBS supplemented with 0.1 % w/v bovine serum albumin (BSA) and the murine monoclonal antibody NCL-HHV-6, that is specific for a 105-kD protein for both HHV-6A and HHV-6B (Novocastra, Newcastle, UK). After washing with PBS, biotinylated horse-anti-mouse Ig antibodies (Vector, Burlingame, CA) were added in PBS supplemented with 1 % w/v BSA and 1 % v/v normal human

serum (NHS) for 45 min at room temperature. After washing with PBS, FITC-conjugated streptavidin (DAKO, Glostrup, Denmark) was added in PBS supplemented with 1 % w/v BSA and 1 % v/v NHS for 45 min at room temperature. Stained cells were embedded in Vectashield mounting medium with 4'6-diamidino-2-phenylindole-2HCl (DAPI; Vector Laboratories, Burlingame, CA) for DNA staining.

Infection of astrocytes using infected T-cell lines

Astrocytes were obtained from post-mortem sub cortical brain white matter provided by the Netherlands Brain Bank after rapid autopsy. Autopsies are performed on donors with written informed consent from the donor or from direct next of kin. Astrocytes were isolated and cultured as described previously (22). Astrocytes derived from two different control donors were used, both free from any clinical or neuropathological signs of CNS disorders. Both donors were females that had succumbed to acute myocardial infarction; donor 1 (00- 022, age 83) and donor 2 (00-031, age 77). Human adult astrocytes were obtained as previously described (23,24). Before addition to astrocyte cultures HHV-6-infected T-cells were resuspended in fresh astrocyte culture medium (DMEM/HAM-F10 supplemented with 10 % FCS and pyruvate, glutamine and antibiotics) to remove soluble mediators or virus released from infected T-cells. Post-confluent monolayers of astrocytes in chamberslides were cultured in the presence of 0.37×10^4, 0.75×10^4, 1.5×10^4 or 3×10^4 HHV-6-infected T-cells per 500 μL for a period of 18 h. Astrocytes were washed extensively to remove the T-cells, fresh culture medium was added and the astrocytes were cultured for an additional period of 1 to 5 days. Infection levels in astrocyte cultures were determined as described above for T-cells.

Preparation of cell-free virus

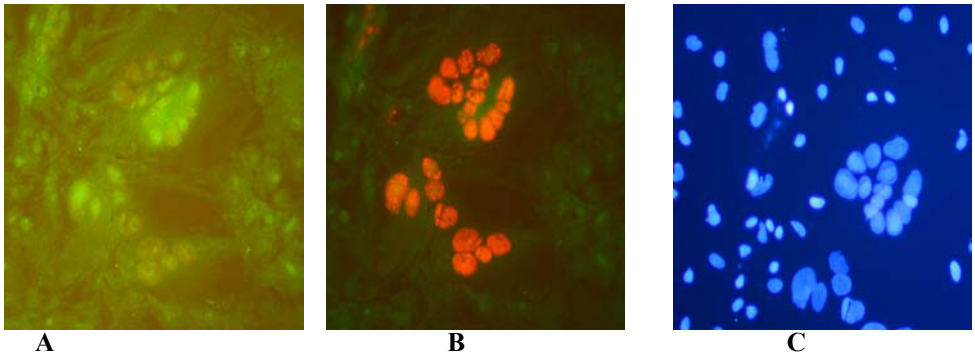
HHV-6-infected HSB-2 and JJHan T-cells were grown for 8 days to maximize viral load. Under these conditions, at least 80 % of the T-cells were infected. Previous experiments indicated that the level of infection as expressed by HHV-6 antigen positivity produced a more reliable measure for infectivity of virus-containing T-cell stocks than the commonly used $TCID_{50}$. Cells were collected by centrifugation at 210 g for 10 min and resuspended in a small part of their original culture supernatants supplemented with 10 % FCS at 1.5×10^6 cells/mL for storage at -80ºC. To prepare cell lysates virus preparations, infected T-cells were subjected to three freezing-thawing cycles. This effectively lysed all cells as verified by re-culturing the lysate. The cell lysates were added to astrocytes at

concentrations of 1.6 x 10^4, 3.2 x 10^4, 6.4 x 10^4 or 12.8 x 10^4 lysed cells per 500 µL and astrocytes were cultured for a period of 1 to 5 days.
As alternatives to the above method to produce a crude total lysate of HHV-6-infected T-cells, additionally purified variant lysates and cell-free HHV-6 preparations (free from any viable cells as well as from cell particles) were prepared by an additional treatment of total lysates including centrifugation at 210 g for 10 min followed by ultracentrifugation of the supernatant (2 h at 60,000 g), filtration over 0.2 or 0.45 µm filters, or by washing of the crude lysate with 150 mM NaCl in the absence or presence of 10 mM ethylenediaminetetra-acetic acid (EDTA).

Results

Infection of astrocytes using HHV-6-infected T-cell lines

Infection of astrocytes was achieved by co-culturing cells with different doses of HHV-6 infected T-cells for 18 h, after which T-cells were carefully removed from the adherent astrocyte population. This led to successful transfer of HHV-6 to cultured astrocytes, as assessed by evaluation of CPE and by staining with an HHV-6 specific antibody (Figure 3.1). Astrocytes were found to be positive for HHV-6 antigen in their nucleus from the first day onward, with CPE such as nucleus swelling and syncytium formation perfectly matching the staining for HHV-6.



A **B** **C**

Figure 3.1. HHV-6-infected astrocytes
HHV-6-infected human adult astrocytes were stained for GFAP (green channel; Figure 3.1.A) and the HHV-6 105-kD protein antigen (red channel; Figure 3.1.B). Cytopathic effects including syncytium formation and swelling of nuclei (DAPI staining; Figure 3.1.C) along with the presence of HHV-6 antigen affects between 30 and 35 % of all astrocytes in culture.

Figure 3.2 illustrates the levels of infection, as expressed by the percentage of HHV-6 positive astrocytes over the course of 5 days after using either HSB-2 (Figure 3.2.A) or JJHan T-cells (Figure 3.2.B) as virus donor cells.

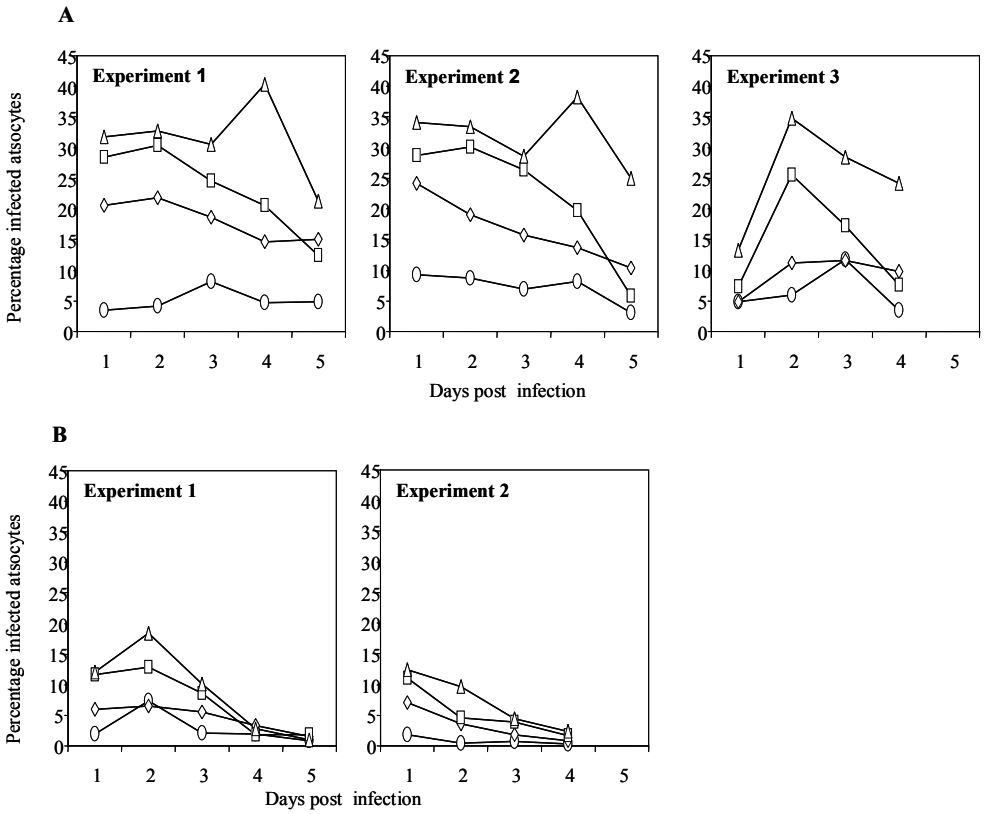


Figure 3.2. Levels of infection in human astrocytes using different HHV-6 donor T-cell lines

○ 0.37 x 10^4 T-cells, ◊ 0.75 x 10^4 T-cells, □ 1.5 x 10^4 T-cells, Δ 3 x 10^4 T-cells.
Cultured astrocytes were supplied with HHV-6 infected HSB-2 T-cells (A) or JJHan T-cells (B) and after an overnight incubation, donor T-cells were carefully removed. For the first 5 days of continued cultures of T-cell free astrocytes, the percentage of infection was determined by specific staining for the common 106 kDa HHV-6 antigen. The experiments, using different doses of donor T-cells, were performed three (A) and two (B) times; results are given for all experiments separately.

Using HSB-2 cells as virus donor cells the percentage of infected astrocytes was found to reach levels of 30-35 % in the first two days of culture. As can be expected, increasing the dose of virus donor cells also increased the level of infection, reaching maximum values with a ratio of T-cells to astrocytes of approximately 40:1 to 100:1. At that level, adding more T- cells did not increase the level of infection (data not shown). From day 3 onward, the percentage of infected astrocytes in the cultures declined. This most likely reflects a combination of more rapid growth of uninfected astrocytes in the culture relative to infected cells, and cell death in infected astrocytes after this period of culturing.
In Table 3.1, the total numbers of astrocytes in the cultures throughout the 5-day culture period are given to illustrate this latter point. The combination of these effects reduced the percentage of infected astrocytes to about 25 % at day 5. Using JJHan T-cells as virus donors, the maximum levels of infection attainable were clearly less, reaching maximum levels of only about 15 % in the first two days (Figure 3.2.B). Also in this case, reduction in the percentage of infected cells was observed upon continued culture, along with increasing levels of cell death after 3 to 5 days following addition of the highest dose of infected donor T-cells (Table 3.2). Using the same conditions, JJHan T-cells were thus clearly less efficient that HSB-2 cells in transmitting HHV-6 to human adult astrocytes.

Table 3.1. Total cell numbers in astrocyte cultures supplied with increasing doses of HHV-6-infected HSB-2 T-cells

culture time (days)	uninfected astrocytes	plus 0.37×10^4 T-cells	plus 0.75×10^4 T-cells	plus 1.5×10^4 T-cells	plus 3×10^4 T-cells
Experiment 1					
1	394	295	315	386	391
2	460	380	343	352	360
3	684	371	306	268	308
4	996	299	341	214	203 †
5	1040	498	315	418	236 †
Experiment 2					
1	366	426	407	383	358
2	507	549	371	408	386
3	811	593	426	391	164 †
4	1198	484	558	263 †	178 †
5	1340	842	571	482	180 †
Experiment 3					
1	1049	877	1004	846	823
2	1047	874	982	970	855
3	1235	1127	1200	982	830
4	1560	1541	1182	1063	785

† = extensive cell death

Table 3.2. Total cell numbers in astrocyte cultures supplied with increasing doses of HHV-6-infected JJHan T-cells

culture time (days)	uninfected astrocytes	plus 0.37×10^4 T-cells	plus 0.75×10^4 T-cells	plus 1.5×10^4 T-cells	plus 3×10^4 T-cells
Experiment 1					
1	394	374	451	464	496
2	460	296	232	479	434
3	684	671	751	638	569
4	996	987	823	782	819
5	1040	1182	1213	1121	327 †
Experiment 2					
1	366	325	312	361	541
2	507	472	440	389	385
3	811	850	798	735	567
4	1198	1128	1075	1020	798

† = extensive cell death

Infection of astrocytes using lysates of HHV-6-infected T-cells

The goal of our study was to establish an astrocyte cell culture model for evaluating the immunomodulatory effects of HHV-6. Cell types other than astrocytes, or products derived from such cells could potentially complicate such as model. Therefore, we examined the possibility to infect astrocytes using a cell-free source of HHV-6. To this end, a lysate was prepared of HHV-6-infected HSB-2 or JJHan T-cells by freezing and thawing the infected cells. After such a treatment, no viable cells could be detected in the lysate as judged by the lack of any T-cell growth under standard culturing conditions. When supplied with such a lysate, about 10 % of the astrocytes displayed CPE typical for HHV-6 infection including swelling of nuclei and syncytium formation and could be successfully stained for nuclear HHV-6 antigen in the first 2 days of culture (data not shown). Staining of infected astrocytes, however, was complicated by widespread staining also of cellular debris non-specifically adhering to astrocyte surfaces. Increasing the doses of the lysate or the culture time did not lead to

any higher levels of infection in the astrocyte cultures. Finally, several additional purification steps were tested for their ability to produce a more infectious lysate of HHV-6 carrying HSB-2 or JJHan T-cells. Ultracentrifugation to selectively collect viral particles, filtration to remove cellular debris, or washing steps to liberate viral particles from subcellular organelles, however, were all found ineffective to produce a lysate or cell-free virus that could infect more than 10 % of cultured astrocytes.

Discussion

The goal of the present study was to obtain a cell culture model that could be used to evaluate the impact of HHV-6 on inflammatory pathways in human adult astrocytes. Such a model is considered useful given the ability of the virus to encode chemokine receptors and chemokine mimics, which could play important roles in inflammatory neurodegenerative disorders such as MS. To establish such a model, a reasonable percentage of the astrocyte culture should be infected over a suitable period of time, without triggering massive cell death. Previously, such massive cell death was reported for HHV-6-infected human fetal astrocytes. To the best of our knowledge, no reports have appeared that document HHV-6 infection of cultured human adult astrocytes.

Using the HHV-6-infected T-cell line HSB-2 as a carrier, a maximum of about 30-35 % of cultured astrocytes could be infected with the virus, as judged by specific staining and virus-induced syncytium formation. This maximum level of infection was achieved with an excess of T-cells over astrocytes in the overnight virus pulse of about 40- to 100-fold. Higher numbers of T-cells led to rapid cell death of astrocytes with detectable infection percentages over the first 2 days of culture still not exceeding 35 %. Also initially lower percentages of infection in the culture did not allow maximum levels to exceed the 35 % maximum by virus spread to other astrocytes (Figure 3.1). A possible increase of infection levels by viral spread, should it have occurred at all, is apparently more than compensated for by the more rapid proliferation of uninfected cells in the culture as compared to infected cells, reducing the overall infection levels again over the course of the first 5 days of culture. Together, these data indicate that a percentage of infected astrocytes of about 35 appears to be the maximum attainable with either of the two donor T-cell lines. Why HSB-2 is markedly more efficient than JJHan in transmission of HHV-6 to astrocytes currently remains unclear.

The use of HHV-6-infected T-cell lysates or cell-free HHV-6 preparations did not lead to levels of infection in astrocyte cultures comparable to those obtained with cell-borne virus. Only about 10 % of the cells could be

infected this way. This only poor performance of these virus preparations is in line with a previous report showing more rapid syncytium formation also in fetal astrocytes with cell-borne *versus* cell-free HHV-6 (15). Possibly, interactions between the donor T-cell line and the recipient astrocyte contribute to productive infection in ways beyond the mere interaction between a viral particle and the HHV-6 receptor CD46.
In conclusion, the use of the HHV-6 carrying T-cell line HSB-2 allows for the experimental infection of about one-third of human adult astrocyte cultures over a period of 2-3 days. After that period, infected cells start to die and overall infection levels decrease. Over these first days, therefore, the impact of HHV-6 on inflammatory pathways in astrocytes can be suitably studied.

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CHAPTER 4

Modulation of the cytokine network in human adult astrocytes by human herpesvirus-6

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Abstract
Human herpesvirus-6A (HHV-6A) is a common pathogen whose role in central nervous system (CNS) disorders including multiple sclerosis remains controversial. To understand how HHV-6A could influence inflammatory pathways in the CNS, we infected cultured human adult astrocytes and examined the expression of 268 cytokines, chemokines, growth factors and their receptors by cDNA array gene profiling.
HHV-6 infection alone had little effect on the astrocyte gene profile but strongly altered the astrocyte response to proinflammatory cytokines. Under those conditions astrocytes express higher levels of anti-inflammatory mediators including IL-10 and IL-11, chemotactic factors, growth factors and factors controlling type I interferon production. Our data suggest that HHV-6 itself does not evoke a pro-inflammatory response in astrocytes but rather triggers immune modulatory factors in the face of inflammation.

Introduction
Human herpesvirus-6 (HHV-6) is a β-herpes virus of which two variants are known, viz. HHV-6A and HHV-6B (1). About 95 % of the human population is persistently infected with HHV-6, with infection usually occurring within the first two years of life (2,3,4). HHV-6 is present in different tissues including mononuclear cells, salivary glands, lungs, genital tract, brain and cerebrospinal fluid (CSF) (1,5,6). In the central nervous system (CNS) HHV-6 can infect astrocytes, oligodendrocytes and microglia (7,8,9,10,11) through CD46, the viral receptor that has been documented on various glial and neural cells (12,13).
HHV-6 has been implicated in clinical manifestations such as exanthem subitum, fever, encephalitis, pneumonitis and hepatitis, and it appears to be an important co-factor in HIV infection (14,15). HHV-6 infection of the CNS is usually silent allowing the virus to persistently remain inside neural cells as a normal CNS commensal. Only occasionally does HHV-6 appear to be associated with neuroinflammation, febrile seizures, encephalitis or encephalopathy (16). For a number of years already, a controversy exists as to the possible involvement of HHV-6 in multiple sclerosis (MS) (17,18). HHV-6 has been detected in oligodendrocytes of MS patients (19) and signs of active HHV-6 infection have been found in CNS tissue, lymphoid tissues, peripheral blood lymphocytes, serum and urine of MS patients (20,21,22). On the other hand, several other studies have failed to produce evidence in support of an association between HHV-6 and MS (23,24,25,26).

Even accepting a possible association between HHV-6 and MS, however, it has remained obscure how exactly HHV-6 could influence its pathogenesis. A striking feature of HVV6 is its ability to modulate inflammatory pathways in target cells. The U12 region of the viral genome encodes a beta chemokine receptor for CCL2, CCL3, CCL4 and CCL5, and the U51 region encodes a CCL5 receptor. Also, a chemokine-like CCR2 agonist is coded for by the viral U83 region (27,28,29,30). By introducing these chemokine (receptor) mimics into the target cell HHV-6 may well impact on the propensity of the infected cell to produce certain mediators or on its response to exogenous stimuli such as pro-inflammatory cytokines or chemokines. In addition, viral interaction with its CD46 receptor may also lead to immune modulatory effects as CD46 ligation influences cytokine production. For example, HHV-6 engagement by CD46 on macrophages suppresses the production of IL-12 that is normally induced by IFN-γ or LPS (31).

To understand how HHV-6 could influence inflammatory processes in the CNS we infected cultured human adult astrocytes and performed gene profiling on 268 cytokines, chemokines, growth factors and their receptors. Apart from the effect of infection itself, we also examined the response of infected astrocytes to additional pro-inflammatory cytokines. Our data indicate that HHV-6 infection alone hardly influences expression of cytokines, chemokines or growth factors. Yet, following additional stimulation with TNF-α, IL-1β or IFN-γ, infected astrocytes respond very differently from uninfected cells. Under those conditions astrocytes produce elevated levels of anti-inflammatory mediators including IL-10 and IL-11, several chemotactic products, growth factors and factors that control type I interferons while uninfected cells do not. These data suggest that HHV-6 infection of astrocytes is unlikely to provoke a host defense response but instead, modulates the cytokine network of astrocytes when these are provoked by inflammation.

Materials and methods

Donors

Astrocytes were obtained from post-mortem sub cortical brain white matter provided by the Netherlands Brain Bank after rapid autopsy (post-mortem delays of less than 10 h). The Netherlands Brain Bank supplies post-mortem specimens from clinically well documented and neuropathologically confirmed cases. Autopsies are performed on donors with written informed consent from the donor or from direct next of kin. All procedures applied in the experiments described in this study were approved by the local ethics committee. Astrocytes derived from two different control donors were used, both free from any clinical or neuropathological signs of CNS disorders. Both donors were females that had succumbed to acute myocardial infarction; donor 1 (00- 022, age 83) and donor 2 (00-031, age 77).

Isolation and infection of human adult astrocytes with HHV-6

The human $CD4^+$ T-lymphoblastoid cell line HSB-2 was used as a carrier cell line for HHV-6A (Strain GS) as described in Chapter 3. Cells were grown in RPMI 1640 (Gibco) supplemented with 10 % FCS and antibiotics at 37 ºC in a humidified atmosphere containing 5 % CO_2. Viral stocks were prepared by freezing HHV-6-infected HSB-2 cells when 75 % HHV-6 positivity was reached as assessed by cytochemical staining with the murine monoclonal antibody NCL-HHV-6 that is specific for a 105-kD protein for both HHV-6A and HHV-6B (Novocastra, Newcastle, UK). Previous experiments indicated that the level of infection as expressed by HHV-6 antigen positivity produced a more reliable measure for infectivity of virus-containing T-cell stocks than the commonly used tissue culture infectivity dose ($TCID_{50}$). Virus stocks were stored in 90 % v/v FCS and 10 % v/v dimethylsulfoxide (DMSO; Merck, Darmstadt, Germany) at -150ºC until further use.

Human adult post-mortem astrocytes were obtained as previously described (32,33). The purity of the astrocyte culture was verified by staining with rabbit anti-glial fibrillary acidic protein (GFAP; ZYMED, San Francisco, CA) as an astrocyte marker, murine anti-human CD68 (DAKO, Glostrup, Denmark) as a microglial marker, and murine anti-myelin basic protein (MBP; Boehringer Mannheim, Germany) as an oligodendrocyte marker. The astrocyte cultures were found to be essentially 100 % GFAP-positive. No cells were found to express CD68 or MBP.

Astrocytes were infected using a HSB-2 carrier cell line. Before adding the infected carrier cells to the astrocytes HSB-2 cells were washed and

resuspended in fresh astrocyte culture medium to eliminate any pre-existing soluble mediators produced by HSB-2. To determine the percentage of infection in cultured astrocytes, target cells were cultured in chamber slides (Falcon, Becton Dickinson, NY) in the presence of 1.5 x 10^4 HHV-6-infected HSB-2 cells per chamber (0.69 cm^2) for a period of 18 h. Astrocytes were extensively washed to remove HSB-2 cells, fresh culture medium was added and the astrocytes were cultured for an additional 48 h in the presence or absence of TNF-α, IL-1β or IFN-γ (PreproTech, Rocky Hill, NY) or a mixture of these cytokines each at a concentration of 500 U/mL, as previously described (32,34). Chamberslides were washed with PBS and cells were fixed in acetone supplemented with 0.03 % v/v H_2O_2 (Merck, Darmstadt, Germany) for 10 min. Chamber slides were air-dried and incubated overnight at 4ºC with rabbit polyclonal antibodies against glial fibrillary acidic protein (GFAP; ZYMED, San Francisco, CA) and NCL-HHV-6 in PBS supplemented with 0.1 % w/v BSA. After washing with PBS biotinylated donkey anti-rabbit Ig antibodies (Amersham Life Science, Roosendaal, The Netherlands) were added in PBS supplemented with 1 % w/v BSA and 1 % v/v normal human serum (NHS) for 45 min at room temperature. After washing with PBS, FITC-conjugated streptavidin (DAKO, Glostrup, Denmark) and TRITC-conjugated donkey anti-mouse Ig (Jackson, West Grove, PA) in PBS supplemented with 1 % w/v BSA and 1% v/v NHS were added for 45 min at room temperature. Slides were embedded in Vectashield mounting medium with 4'6-diamidino-2-phenylindole-2HCl (DAPI; Vector Laboratories, Burlingame, CA) for DNA staining.

To prepare astrocytes for gene profiling, post-confluent astrocyte cultures were supplemented with 1.6 x 10^6 HHV-6-infected HSB-2 cells per 75 cm^2 flask for a period of 18 h. Astrocytes were extensively washed to remove HSB-2 cells, fresh culture medium was added and the astrocytes were cultured for an additional 48 h in the presence or absence of TNF-α, IL-1β and IFN-γ (PreproTech, Rocky Hill, NY) each at a final concentration of 500 U/mL. Uninfected and untreated astrocyte cultures were used as controls.

mRNA profiling using cDNA arrays

Analysis of the mRNA profile of astrocytes was performed by hybrid selection of radioactively labeled cDNA on high-density Clontech Atlas® arrays of membrane-bound cDNA probes, as described previously (34). Briefly, for each condition total cellular RNA was extracted from the astrocytes and poly A^+ RNA was separated from total RNA. Radioactive

cDNA probes were prepared and hybridization of the probes to the membranes was carried out overnight. The hybridization signal for each gene probe, present in duplicate on the array, was calculated as the mean of these duplicates, corrected for background intensity and quantified using software provided by the manufacturer. Relative hybridization signals were calculated by dividing the background-corrected hybridization signals for each gene by the mean signal for all nine housekeeping reference genes on the corresponding array, and multiplying this ratio by 1,000. When comparing gene expression signals between different samples, ratios were calculated by dividing the relative expression signals. To avoid extreme ratio values, a minimum of 10 was applied to very low relative expression signals.

Results

HHV-6 infection of cultured human adult astrocytes

As a first step in this study, we verified whether experimental infection of cultured astrocytes with HHV-6 led to a reproducible and stable level of infection over the course of the experiments. In this study, we chose to work with the HHV-6A variant as this strain is more frequently detected in CSF of children and adults and thus, may have a greater neurotropism that the B strain (35). Infection of astrocytes was established by co-culturing the cells with an HHV-6A-infected HSB-2 carrier T- cell. Attempts to infect astrocytes using cell-free preparations such as culture supernatants or cell lysates of HSB-2 cells were unsuccessful (see Chapter 3). To monitor the percentage of infected astrocytes following co-culture with infected HSB-2 cells cytopathic effects (CPE) including enlargement of nuclei, cell swelling and syncytium formation was evaluated. These CPE were very similar to those previously described by others for HHV-6-infected fetal astrocytes, oligodendrocytes and microglia in culture (7). Also, astrocytes were stained for the 105-kDa antigen common to both the HHV-6A and HHV-6B strain. As a confirmation that CPE were the direct consequence of HHV-6 infection the HHV-6 105-kDa antigen was found to be consistently present in cells with marked CPE (Figure 3.1 of Chapter 3) and not in normal-appearing astrocytes. It should be noted, however, that not all stages of the HHV-6 infection cycle may be reflected by CPE and/or the presence of sufficient levels of the 105-kDa to allow for immunocytochemical staining. In view of the lack of markers for very early stages of HHV-6 infection, levels of infection in the culture as assessed by CPE and staining for the 105-kDa antigen therefore only represent minimum values for the level of infection in astrocyte cultures. Over the 48-h culture time of the

experiments, between 30 % and 35 % of all astrocytes were visibly affected by HHV-6 infection. Infection levels at the start of the experiment were very similar to those observed after 48 h indicating that viral spread in the absence of the original HSB-2 carrier cell line was negligible. Supplementing astrocyte cultures with TNF-α, IL-1β or IFN-γ had no detectable influence on the percentage or morphology of HHV-6-infected astrocytes (data not shown).

Gene profiling of HHV-6-infected astrocytes

Both HHV-6-infected and uninfected astrocyte cultures were evaluated for their gene expression profiles after 48 h by cDNA array gene profiling as previously described by us (34). In this analysis which was performed three times using different astrocyte cultures, 268 genes encoding cytokines, chemokines, growth factors and their receptors were monitored. Astrocytes from donor 1 were used for experiment 1, while astrocytes from donor 2 were used for experiments 2 and 3. The data consistently revealed only very minor effects of infection, as illustrated in Figure 4.1 and summarized in Table 4.1. Only four genes represented on the array were reproducibly induced more than two fold by HHV-6 infection. These included leukocyte interferon-inducible peptide (LIP), CCL5 (RANTES), insulin-like growth factor binding protein 6 (IGFBP6) and vascular endothelial growth factor C (VEGF-C). Apart from CCL5, therefore, no distinct pro-inflammatory products appeared in the HHV-6-infected astrocyte cultures that would be expected to promote a host-defense response. As the result of HHV-6 infection, some genes were reproducibly suppressed at least two fold. These included trk-T3, IGFBP complex acid labile chain (IGFBP), ephrin type-A receptor 5, ephrin-B2, transforming growth factor-beta 3 (TGF-β3), colon carcinoma kinase 4 (CCK4) and transmembrane receptor PTK7. Yet, these effects were minor as expression levels of all these products were already rather low in control cultures.

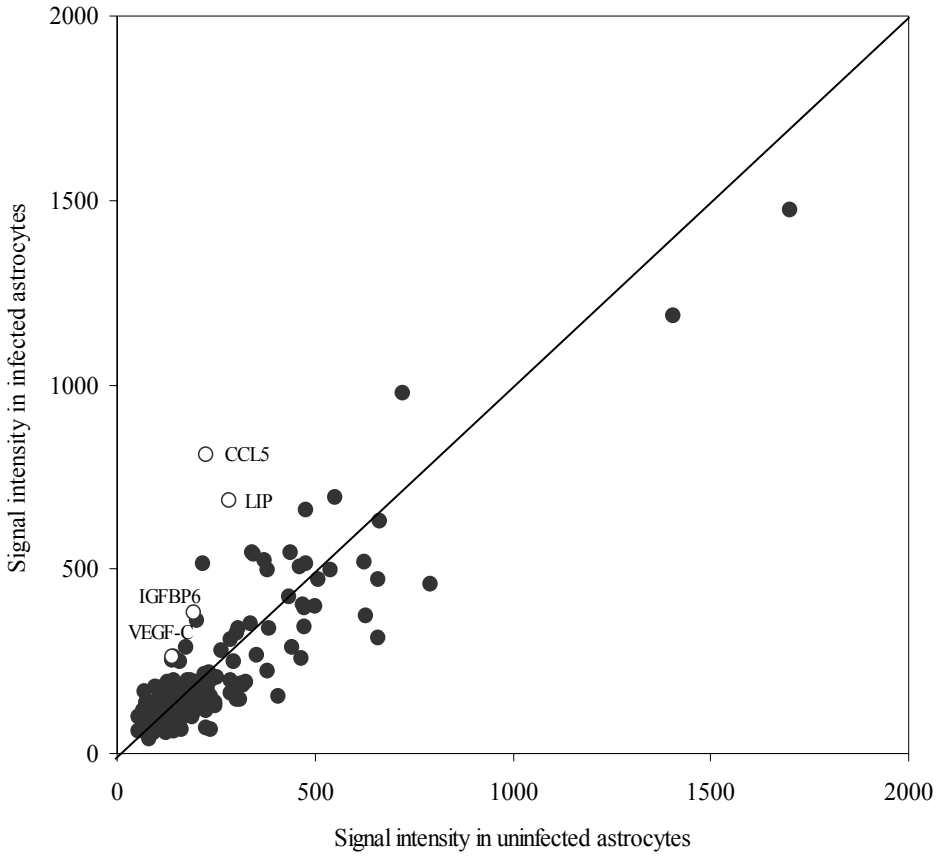


Figure 4.1. The impact of HHV-6 infection on the cytokine network of astrocytes
Astrocytes were infected with HHV-6 and after 48 h expression levels of 268 genes encoding cytokines, chemokines, growth factors and their receptors were compared to those in uninfected control astrocytes. Signals at or around the diagonal represent genes whose expression is unaffected by HHV-6 infection. Signals in the upper left part represent genes whose expression is enhanced by the infection while signals in the lower right part represent genes whose expression is inhibited. Data are shown from a single representative experiment.

Table 4.1. Genes with altered expression in HHV-6-infected astrocytes.

Gene name	Uninfected			HHV-6 infected			Mean ratio	Mock infected ratio	Gene code
	Exp 1	Exp 2	Exp 3	Exp 1	Exp 2	Exp 3			
induction									
leukocyte interferon-inducible peptide	285	38	455	685	539	1026	6.3	1.0	X02492
CCL5 (RANTES)	226	377	321	809	332	741	2.3	0.9	M21121
insulin-like growth factor binding protein 6 (IGFBP6)	193	<10	133	380	252	275	9.8	0.7	M62402
vascular endothelial growth factor C (VEGF-C)	139	<10	79	260	284	237	11.1	0.6	U43142
suppression									
trk-T3	191	114	405	100	<10	275	0.4	0.9	X85960
IGFBP complex acid labile chain	128	275	120	105	<10	61	0.5	1.1	D25216
ephrin type-A receptor 5	174	253	145	107	<10	52	0.3	0.3	X95425
ephrin-B2	75	207	106	93	<10	48	0.3	0.3	L38734
transforming growth factor-beta 3 (TGF-β3)	115	111	136	69	<10	13	0.3	0.9	J03241
colon carcinoma kinase 4 + transmembrane receptor PTK7	120	105	85	58	<10	<10	0.3	0.7	U33635 + U40271

Genes are listed when their reference expression reached relative values of at least 100

To verify whether the above effects were indeed caused by the infection itself and did not result from the presence of residual carrier T-cells in the culture system, the effect of adding uninfected instead of infected HSB-2 carrier T-cells on the astrocyte gene profile was also evaluated. The effect of the HSB-2 carrier itself on astrocytes turned out to be minimal and only the expression of ephrin type-A receptor 5 and ephrin-B2 were somewhat suppressed, both with a factor of 3 (Table 4.1). All other genes that were induced or suppressed by infection remained unaffected in their expression following addition of only the carrier cell line.

Gene profiling of HHV-6-infected astrocytes upon a pro-inflammatory insult

Next, we investigated the effects on infected astrocytes of a mixture of TNF-α, IL-1β and IFN-γ. The combination of HHV-6 infection and these cytokines induced extensive and frequently very strong alterations in the gene profile after 48 h, as illustrated in Figure 4.2.

Tables 4.2 and 4.3 present the changes in gene expression levels with regard to genes that were either induced or suppressed in their expression at least two fold. The gene profile of infected and treated astrocytes can be compared to the gene profile of uninfected treated astrocytes as illustrated in Figure 2.4 of Chapter 2.

In HHV-6-infected astrocytes the pro-inflammatory cytokines induced markedly elevated levels of IL-10, IL-11, IL-1β, and IL-6. Especially the induction of the anti-inflammatory cytokines IL-10 (4-fold) and IL-11 (24-fold) is striking since in uninfected astrocytes no IL-10 is induced at all, and IL-11 only 3-fold. Secondly, TNF-α, IL-1β and IFN-γ induced expression of several chemotactic products in HHV-6-infected astrocytes. Whereas uninfected astrocytes show strongly elevated levels only of CXCL8 (IL-8) in response to the pro-inflammatory cytokines, HHV-6-infected astrocytes displayed induction also of CCL3 (MIP-1α), CXCL2 (MIP-2α), CCL5 (RANTES) and CXCL6 (granulocyte chemotactic protein 2) along with CXCL8.

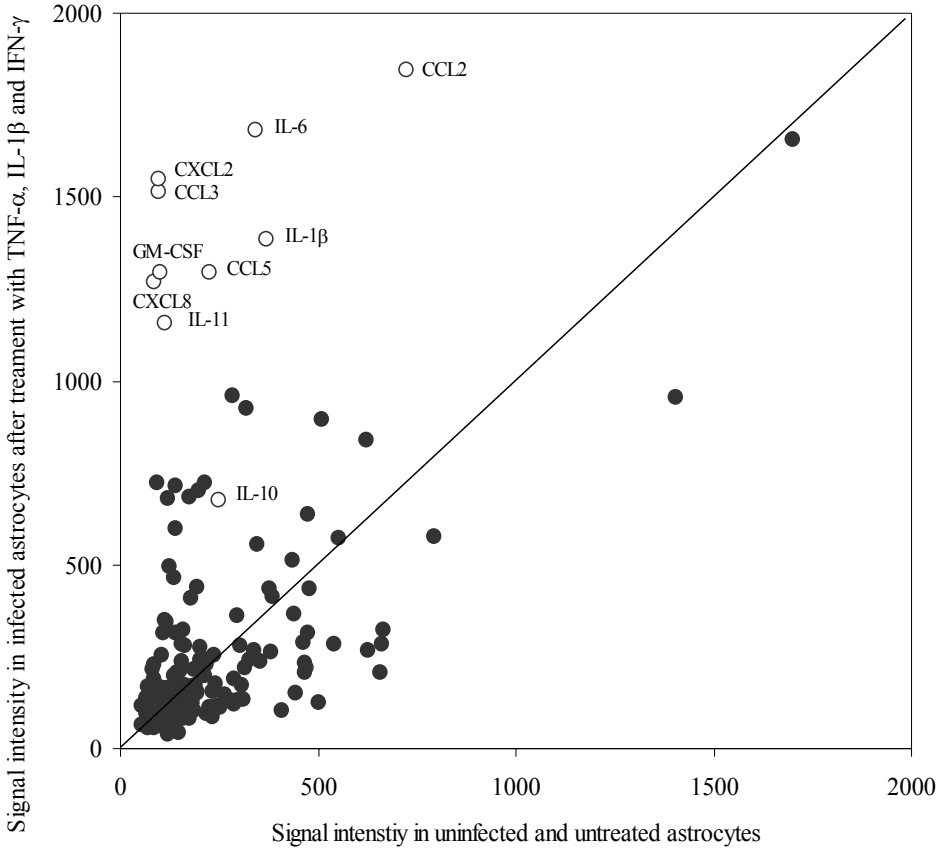


Figure 4.2. The impact of pro-inflammatory cytokines on the cytokine network of HHV-6-infected astrocytes

HHV-6-infected human adult astrocytes were treated with a mixture of TNF-α, IL-1β and IFN-γ and after 48 h expression levels of 268 genes encoding cytokines, chemokines, growth factors and their receptors were compared to those in uninfected and untreated control astrocytes. Signals at or around the diagonal represent genes whose expression is unaffected by cytokine treatment. Signals in the upper left part represent genes whose expression is enhanced by the cytokines while signals in the lower right part represent genes whose expression is inhibited.

Table 4.2. Genes induced after treatment of HHV-6-infected astrocytes with TNF-α, IL-1β and IFN-γ

Gene name	HHV-6 infected		
	Exp 1	Exp 2	Exp 3
CCL2 (MCP-1)	722	580	788
interleukin-6 (IL-6)	342	163	429
CCL5 (RANTES)	226	377	321
CXCL8 (IL-8)	84	<10	51
CXCL2 (MIP2-α)	97	<10	65
interleukin-1 beta (IL-1β)	371	465	375
CCL3 (MIP1-α)	96	59	91
leukocyte interferon-inducible peptide (LIP)	285	38	455
interleukin-11 (IL-11)	111	<10	121
granulocyte-macrophage colony stimulating factor (GM-CSF)	100	187	49
metallothionein-III (MT-III), growth inhibitory factor	214	33	411
ERBB-3 receptor protein-tyrosine kinase (epidermal growth factor receptor)	319	215	256
interferon regulatory factor 1 (IRF1)	158	173	280
CXCL6 (granulocyte chemotactic protein 2, GCP2)	120	<10	27
vascular endothelial growth factor C (VEGF-C)	139	<10	79
leukemia inhibitory factor (LIF)	140	79	172
insulin-like growth factor binding protein 6 (IGFBP6)	193	<10	133
heparin-binding EGF-like growth factor (HBEGF)	198	<10	77
interleukin-10 (IL-10)	248	27	229
granulocyte colony-stimulating factor (G-CSF)	93	190	59
vascular endothelial growth factor (VEGF)	114	<10	88
neuregulin + neu differentiation factor + heregulin α	109	<10	47
tumor necrosis factor (TNF-α)	118	330	71
erythroid differentiation protein (EDF)	118	40	83
interferon consensus sequence-binding protein (ICSBP)	67	<10	<10
cytokine receptor EBI3	85	316	63
neuregulin	69	<10	20
CD114 (GCSF-R)	83	<10	54
interleukin-7 receptor alpha subunit (IL-7R-α)	105	<10	52
endothelin receptor type B (EDNRB)	56	<10	46

Genes are listed when their expression reached relative values of at least 100 in two of the three experiments

HHV-6 infected plus cytokines			Mean ratio	Gene code
Exp 1	Exp 2	Exp 3		
1844	1487	1677	2.4	M24545
1684	1162	1512	5.2	X04602
1293	1254	1363	4.4	M21121
1269	938	1471	45.8	Y00787
1547	1043	1064	45.6	X53799
1384	1035	1213	3.1	K02770
1516	1056	745	14.0	M23452
958	615	1012	7.3	X02492
1155	561	725	24.2	M57765
1296	443	699	9.9	M11220
723	836	824	10.2	D13365
926	345	715	2.4	M29366
321	702	918	3.1	X14454
679	386	853	25.4	X78686
597	449	812	19.9	U43142
716	269	710	4.2	X13967
438	404	596	15.7	M62402
699	198	427	9.6	M60278
675	193	446	4.0	M57627
722	118	311	4.6	X03438
348	240	546	11.1	M32977
315	103	350	6.9	L12260+U02326+ M94165
343	165	234	2.2	X01394
162	139	299	2.8	J03634
138	181	188	13.2	M91196
229	70	207	2.1	L08187
168	108	198	7.7	L12261
213	20	228	3.4	M59818
95	104	202	5.1	M29696
118	<10	113	2.3	L06623

Table 4.3. Genes downregulated after treatment of HHV-6-infected astrocytes with TNF-α, IL-1β and IFN-γ.

Gene name	HHV-6 infected			HHV-6 infected plus cytokines			Mean ratio	Gene code
	Exp 1	Exp 2	Exp 3	Exp 1	Exp 2	Exp 3		
connective tissue growth factor (CTGF)	659	622	799	207	293	555	0.5	M92934
CCCKR2 (CCR2, monocyte chemoattractant protein 1 receptor)	501	615	786	123	189	575	0,4	U03882
vascular endothelial growth factor receptor 1 (VEGFR1)	628	521	573	268	127	482	0.5	X51602 + U01134
SL cytokine (FLT3 ligand)	229	292	440	94	102	363	0.5	U04806
ephrin type-A receptor 1	444	371	281	151	106	173	0.4	M18391
CD135 (stem cell tyrosine kinase 1, STK1)	305	140	354	131	47	249	0.5	U02687
trk-T3	191	114	405	155	40	151	0.5	X85960
jagged 1 (HJ1)	408	260	456	105	<10	203	0.3	AF028593
sonic hedgehog (SHH)	166	172	217	90	<10	211	0.5	L38518
CD140B (platelet-derived growth factor receptor beta subunit)	288	170	389	129	27	128	0.3	M21616
ephrin type-A receptor 5	174	253	145	82	63	85	0.4	X95425
IGFBP complex acid labile chain	128	275	120	133	<10	48	0.5	D25216
CD117 (proto-oncogene tyrosine-protein kinase kit)	150	127	145	145	13	30	0.4	X06182
macrophage-stimulating protein (MSP)	149	180	157	100	<10	75	0.4	M74178
transforming growth factor-beta 3 (TGF-beta3)	115	111	136	84	<10	55	0.4	J03241
neurotrophin-4 (NT4) + NT5 + NT6-α + NT6-β + NT6-γ	91	310	110	76	15	53	0.3	M86528 + S41522 + S41540 + S41541
ephrin-B2	75	207	106	107	<10	<10	0.5	L38734
TNF-related apoptosis inducing ligand (TRAIL)	158	161	82	80	26	<10	0.3	U57059

Genes are listed when their reference expression reached relative values of at least 100 in two of three experiments.

In addition, the pro-inflammatory stimulus triggered a range of gene products that play roles in regulation of cellular growth and differentiation and that were different from those induced in uninfected cells. These included IGFBP6, VEGF and VEGF-C, granulocyte-macrophage colony-stimulating factor (GM-CSF), neuregulin, heparin-binding EGF-like growth factor (HBEGF), leukemia inhibitory factor (LIF), metallothionein III (MT-III) and the IL-7 receptor α chain. Also induction of granulocyte colony-stimulating factor (G-CSF) and its receptor was monitored, but this induction was also found in uninfected cells. Finally, several products were triggered that control interferon type I production. These included interferon regulatory factor-1 (IRF1), interferon consensus sequence binding protein (ICSBP) and LIP. None of these genes were induced by the cytokines in uninfected astrocytes.

Together, these data clarify that several marked differences exist between the response of HHV-6-infected astrocytes to the pro-inflammatory insult as compared to uninfected astrocytes. In Figure 4.3, these differences are summarized with regard to genes whose expression was enhanced the strongest under the various conditions (induction by at least a factor of 3). To emphasize the difference in the response of HHV-6-infected astrocytes as compared to uninfected cells, several genes were found to be suppressed in their expression by TNF-α, IL-1β and IFN-γ, whereas no genes at all are suppressed in uninfected cells (compare Figures 4.2 and 2.4 of Chapter 2). Suppression, as summarized in Table 4.3, affected among others connective tissue growth factor, Flt3 ligand (SL cytokine) and receptors for CCL2, VEGF and ephrin type A, gene products that are normally expressed at appreciable levels in cultured astrocytes.

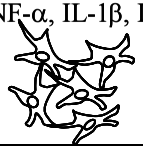
HHV-6	HHV-6 plus TNF-α, IL-1β, IFN-γ	TNF-α, IL-1β, IFN-γ
		
Interferon response regulating genes		
leukocyte interferon-inducible peptide (LIP)	leukocyte interferon-inducible peptide (LIP) interferon regulatory factor 1 (IRF1) interferon consensus sequence binding protein (ICSBP)	
Chemotactic genes		
	CXCL8 (IL-8) CCL3 (MIP-1α) CXCL2 (MIP-2α) CCL5 (RANTES) CXCL6 (granulocyte chemotactic protein 2)	CXCL8 (IL-8)
Growth related genes		
insulin-like growth factor binding protein 6 (IGFBP6) vascular endothelial growth factor C (VEGF-C)	insulin-like growth factor binding protein 6 (IGFBP6) vascular endothelial growth factor C (VEGF-C) vascular endothelial growth factor (VEGF) G-CSF and CD114 (G-CSF receptor) GM-CSF neuregulin heparin-binding EGF like growth factor (HBEGF) IL-7Rα leukemia inhibitory factor (LIF) metallothionein (MT-III)	erythropoietin receptor (EPOR) insulin-like growth factor II (IGF2) amphiregulin G-CSF and CD114 (G-CSF receptor) erythroid differentiation protein (EDF)
Immune modulatory genes		
	IL-11 IL-10 IL-6 IL-1β	IL-11 IL-17 vitamin B3 receptor (G-protein-coupled receptor HM74)

Figure 4.3.
The impact of HHV-6 on the cytokine network of astrocytes either in the absence or presenceof pro-inflammatory cytokines
A comparison between the very different sets of genes that are induced under the various conditions in astrocytes by at least a factor of 3.

To extend the information on the effects of pro-inflammatory cytokines on HHV-6-infected astrocytes, effects were also examined of the individual cytokines in the mixture of TNF-α, IL-1β and IFN-γ. The results of these analyses are summarized in Table 4.4. Six genes emerged as common responders in HHV-6-infected astrocytes to all three cytokines individually. These common responders included IL-11, VEGF-C, LIP and MT-III, IL-6 and CD124 (the IL-4 receptor α subunit). Five out of these six were also found after treatment with all three cytokines together (marked in italics in Table 4.4). Other genes such as CXCL8, HBEGF, CCL3, CXCL2, LIF and neuromodulin (GAP-43) were induced by both TNF-α and IL-1β alone but not by IFN-γ. Genes only induced by IFN-γ not only included the five above-mentioned common response genes but also IGFBP6, endothelial monocyte activating polypeptide II, VEGF, fibroblast growth factor 5 and, interestingly, the IFN-γ receptor β subunit. In itself, the observation that IFN-γ has any effect at all already emphasizes the difference between HHV-6-infected and uninfected astrocytes since in the latter, no effect whatsoever could be monitored of only IFN-γ. However, HHV-6 infection alone had no effect on the expression levels of the IFN-γ receptor. These data suggest that the uninfected astrocytes are in some way blocked to respond to IFN-γ treatment and that infection of astrocytes with HHV-6 dissolves this blockade. The intracellular mechanisms which might be responsible are still unknown.

Most of the genes induced by the individual cytokines as listed in Table 4.4 explain the induction of genes by the mixture of cytokines as listed in Table 4.2. Of all gene products that were induced at least 10-fold in HHV-6-infected cells by the mixture of cytokines only GM-CSF, CXCL6 and ICSBP were not found to be induced by any of the individual cytokines. Thus, the induction only of these three products may be triggered by synergistic effects between the cytokines but for the majority of products induced, such effects appear to be limited, similar to what we previously observed for uninfected astrocytes (34).

Table 4.4. Genes induced after treatment of HHV-6-infected astrocytes with TNF-α, IL-1β or IFN-γ separately.

TNF-α	Ratio	IL-1β	Ratio	IFN-γ	Ratio
interleukin-11	*30.1*	*interleukin-11*	*48.1*	*interleukin-11*	*44.8*
vascular endothelial growth factor C	*29.2*	*vascular endothelial growth factor C*	*40.5*	*vascular endothelial growth factor C*	*32.1*
interleukin-6	*4.1*	*interleukin-6*	*6.4*	*interleukin-6*	*3.1*
leukocyte interferon-inducible peptide	*14.4*	*leukocyte interferon-inducible peptide*	*17.3*	*leukocyte interferon-inducible peptide*	*12.6*
metallothionein-III	*13.9*	*metallothionein-III*	*15.5*	*metallothionein-III*	*11.1*
CXCL8	*55.7*	*CXCL8*	*64.7*	pleiotrophin	3.6
CCL3	*13.1*	*CCL3*	*14.3*	*Interferon regulatory factor 1*	*4.6*
CXCL2	*42.4*	*CXCL2*	*94.9*	*insulin-like growth factor binding P6*	*45.4*
leukemia inhibitory factor	*4.2*	*leukemia inhibitory factor*	*5.3*	interleukin-1 receptor type I	9.8
heparin-binding EGF-like growth factor	*22.5*	*heparin-binding EGF-like growth factor*	*28.6*		
neuromodulin (GAP-43)	3.1	neuromodulin (GAP-43)	5.8		
interleukin-2	3.1	follistatin-related protein	5.2		
CD124 (IL-4 receptor alpha subunit)	6.7	*vascular endothelial growth factor*	*23.6*		
CCL5	*3.3*	interleukin-13	20.4		

Genes are listed when their expression reached relative values of at least 100 under either condition.
Genes in italics are found induced also after treatment with combined cytokines as listed in Table 4.2.

Gene profiling of HHV-6-infected HSB-2 T-cells

Finally, we evaluated the gene profile of the carrier HSB-2 T-cell line that was used to infect astrocytes, as well as that of the HHV-6-infected T-cell line. The gene profile of HHV-6-infected HSB-2 cells is relevant to evaluate the extent of possible contamination of infected astrocyte cultures by RNA derived from the carrier T-cells. Also, it is of interest to compare the impact of HHV-6 on this T-cell line with its impact on astrocytes.

A comparison between the most abundant gene products in the infected HSB-2 cell line with the gene products induced in infected astrocytes either in the presence or absence of pro-inflammatory cytokines indicates that contamination by T-cell derived RNA is highly unlikely to have affected our data. Of the 30 most abundant gene products in the HHV-6-infected HSB-2 cells, only one, viz. LIP, was among the products found to be elevated in the infected astrocyte cultures. Only for this single gene product, therefore, we cannot formally exclude the possibility that its induction could be caused by contaminant HSB-2-derived mRNA. For all other gene products, such contamination appears highly unlikely as an explanation for their induction in astrocytes.

Furthermore, a comparison between the genes induced in HSB-2 by HHV-6 infection with those induced in astrocytes reveal that there is very little similarity in the response by either cell type. Of all genes induced by a factor of at least 3 (Table 4.5), only the response of VEGF-C corresponded with what was observed in astrocytes. All other gene responses induced by HHV-6 in the HSB-2 T-cell line, including suppression of CXCL8, were different from those induced in astrocytes. Clearly, the effects of HHV-6 on expression levels of elements of the cytokine network are highly dependent on the type of target cell.

Table 4.5. Genes with altered expression in HHV-6-infected HSB-2 T-cells

Gene name	uninfected	HHV-6 infected	Ratio	Gene code
induction				
neuregulin + neu differentiation factor + heregulin alpha	<10	178	17.83	L12260 + U02326 + M94165
ephrin type-A receptor 3 (tyrosine kinase receptor HEK)	<10	175	17.48	M83941
frizzled 5	<10	171	17.11	U43318
insulin-like growth factor binding protein 5 (IGFBP5)	<10	156	15.63	M65062
migration inhibitory factor-related protein 8 (MRP8)	21	218	10.35	X06234
ciliary neuronotrophic factor (CNTF) isoforms B & C	54	356	6.65	A26792
interferon-gamma receptor (IFNR-gamma)	25	151	6.10	J03143
epidermal growth factor receptor (EGFR)	33	154	4.65	X00588
low-affinity nerve growth factor receptor (NGFR)	173	788	4.56	M14764
competitive hepatocyte growth factor antagonist (HGF antagonist)	35	156	4.50	M77227
vascular endothelial growth factor (VEGF)	54	226	4.21	M32977
vascular endothelial growth factor C (VEGF-C)	39	163	4.16	U43142
CCL2 (MCP-1)	121	459	3.81	M24545
insulin-like growth factor binding protein 4 (IGFBP4)	48	170	3.58	M62403
interleukin-1 receptor antagonist protein (IL-1RA)	204	682	3.34	M63099
endothelin 3 (ET3)	66	214	3.24	J05081
hepatocyte growth factor (HGF)	52	163	3.17	M60718
suppression				
CXCL8 (IL-8)	410	148	0.36	Y00787

Genes are listed when their expression reached relative values of at least 150 under either condition

Discussion

In this study, gene profiling was used to examine the impact of HHV-6 infection on the cytokine network in cultured human adult astrocytes. To the best of our knowledge, ours is the first attempt to document how infection of human glial cells by HHV-6 may influence inflammatory pathways. Especially for a better understanding of its suggested association with inflammatory neurodegenerative disorders such as MS it is important to appreciate whether the virus could be expected to elicit a pro-inflammatory reaction in the human CNS, or perhaps modulates such reactions. At least with regard to infection of astrocytes the latter appears to be more likely.

As a standard read-out tool in this study we used cDNA arrays which we have previously validated as a comprehensive assay that produces reliable and reproducible results when applied to cultured human adult astrocytes (34). Infection of astrocytes with HHV-6 affected expression of a surprisingly limited number of the 268 genes examined. Only CCL5, LIP, IGFBP6 and VEGF-C were induced whereas all other gene expression levels remained very similar. In sharp contrast, treatment of HHV-6-infected astrocytes with a mixture of TNF-α, IL-1β and IFN-γ resulted in a response that was markedly different from that of uninfected astrocytes both in a quantitative and a qualitative manner. Especially the 4-fold induction of IL-10 and the 24-fold induction of IL-11 by the cytokine mixture is of interest since both are well-known anti-inflammatory cytokines (36,37). IL-11 is only induced 3-fold in uninfected astrocyte cultures while IL-10 is not induced at all. Interestingly, IL-10 has also been found induced in monocytes as the result of HHV-6 infection, ultimately causing IFN-γ-induced downregulation of IL-12 in these cells (31). In astrocyte cultures treated with TNF-α, IL1β or IFN-γ separately, IL-11 consistently emerges as one of the most strongly induced genes with levels of induction between 30- and 50- fold. In uninfected astrocytes, no induction of IL-11 was recorded in response to any of these individual cytokines.

Another gene product that is consistently induced either by individual or mixed pro-inflammatory cytokines is VEGF-C, a growth factor involved in angiogenesis. While already somewhat elevated in HHV-6-infected astrocytes, TNF-α, IL-1β and IFN-γ trigger a further 20- to 40-fold increase in expression levels of VEGF-C which it is not induced by either cytokine in uninfected astrocytes, nor by a mixture of the three cytokines. The related VEGF to which the same applies, is induced 10- to 20-fold, except by TNF-α. Other factors that are induced by the pro-inflammatory cytokines include

the pleiotropic cytokine IL-6 (38,39) and LIP as well as MT-III, both factors whose functions in the CNS remains to be fully established.
It is remarkable that several of the changes effected by HHV-6 is the induction in astrocytes of anti-inflammatory and angiogenic factors in response to an inflammatory stimulus. Also chemotactic factors appear to be induced more prominently by pro-inflammatory cytokines in HHV-6-infected astrocytes as compared to uninfected cells. Among the chemotactic products readily induced in infected cells are CXCL8, CXCL2, CCL3 and CXCL6. Whereas these chemokines, as well as CCL5 (RANTES) that is induced in unstimulated HHV-6-infected astrocytes, are well known to be relevant to leukocyte and granulocyte recruitment it should be pointed out that they are also relevant to glial cells themselves. Both human adult astrocytes, neurons and microglia express receptors for all these chemokines and under the influence of TNF-α astrocytes express even higher levels of CCR3 (receptor for CCL5) and CXCR1 (receptor for CXCL6 and CXCL8) (40). Thus, the chemokine response modulated by HHV-6 should be considered relevant neural cell migration within the CNS as well. Finally, it is interesting to note induction in HHV-6-infected astrocytes but not in uninfected cells of LIP, IRF-1 and ICSBP all of which play a role in controlling production of type I interferons. Such a response appears to be fully consistent with induction of an antiviral response in the target cells as a component of the total response, which is obviously not unexpected.
The effects of HHV-6 on astrocytes appear to be cell specific as the virus has a completely different impact on the gene profile of the HSB-2 carrier T-cell line used by us to infect astrocytes. This profile was examined primarily to exclude the possibility that contaminant RNA from this cell line could have affected the results. At the same time, our analysis reveals that while HHV-6 hardly impacts on astrocytes, the virus does trigger induction of several genes in HSB-2 cells. All of these, except for VEGF-C are different from those induced in astrocytes. When compared to a previous report on the effect of HHV-6 on another T cell line, viz. SupT1 (41) differences are again seen. In SupT1 HHV-6 induced IL-18 and the IL-2 receptor and led to suppression of IL-10, the IL-10 receptor and IL-14. None of these changes could be observed in the presently examined cell line HSB-2, despite that fact that in both cases the A strain of HHV-6 was used. Together, these data underpin the notion that HHV-6 can have very different effects in different types of cells.
In summary, HHV-6 appears unlikely to trigger an immediate host-defense response in astrocytes which is consistent with its frequent and silent infection of the human CNS. In the face of a pro-inflammatory stimulus, however, the presence of HHV-6 in astrocytes profoundly alters their

response and leads to elevated expression of anti-inflammatory mediators, chemokines and pro-angiogenic factors. This appears to be in line with the recent observation of immunosuppressive effects of HHV-6 infection also on macrophages, in which the virus blocks inflammation-induced IL-12 production (31). Clearly, HHV-6 may have additional effects that are relevant to inflammatory demyelinating diseases including for example its negative effect on proliferation and development of glial precursors as recently observed *in vitro* (42). Based on its effect on astrocytes, however, HHV-6 appears unlikely to help trigger a local inflammatory reaction in the CNS but rather promotes downregulation of such a reaction.

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CHAPTER 5

Infection of human adult astrocytes with human herpesvirus-6 induces elevated expression of Toll-like receptors but does not activate them

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Abstract
Toll-like receptors (TLR) are key players in innate immune responses in a wide range of cell types and tissues including the central nervous system (CNS). In particular during inflammation microglia and astrocytes in the human brain express marked levels of TLR. To explore their role in the host response to neurotropic infections we examined the impact of human herpesvirus-6 (HHV-6) on TLR expression in cultured astrocytes. HHV-6 is a frequent pathogen in the human adult CNS but does not usually trigger inflammatory reactions in the brain or spinal cord.
Astrocytes derived from post-mortem human adult brain samples were infected with HHV-6 in culture and expression levels of TLR1 to TLR4 were monitored by real-time quantitative PCR and cytokine production was monitored via gene profiling. Also, the effects of the pro-inflammatory cytokines IL-1β, TNF-α and IFN-γ on TLR expression were examined, both in HHV-6 infected and uninfected astrocytes.
HHV-6 infection of astrocytes led to a modest increase in levels of mRNA encoding the dominant TLR4 or the less abundant TLR1 and TLR2, but to a strong increase in levels of TLR3-encoding mRNA. Similar effects were observed after treatment of astrocytes with pro-inflammatory cytokines. The combination of HHV-6 infection and pro-inflammatory cytokines again led to strong induction of TLR3 but in this case, also TLR2 was induced between 10- to 100-fold. Interestingly, HHV-6 infection of astrocytes did not lead to production of any significant amounts of IL-1β, TNF-α, IL-6 or other cytokines traditionally marking TLR-mediated responses. These results suggest that while HHV-6 does induce elevated expression especially of TLR3, it does not appear to provide the ligands necessary to activate a TLR-mediated response in astrocytes.

Introduction

The family of mammalian Toll like receptors (TLR) play key roles in innate responses against invading pathogens (1,2). TLR mediate responses to a wide range of pathogen-associated products including lipopolysaccharides, lipoproteins, flagellin, bacterial DNA and double-stranded RNA. Such responses typically involve production of a range of inflammatory mediators including TNF-α, IL-1β, IL-6, nitric oxide and type I interferons (3). Expression of several TLR family members has been documented also for the human CNS (4). TLR are particularly prominent on glial cells and their expression is elevated during CNS inflammation. In cell culture models human adult microglia express a wide range of different TLR, predominantly inside intracellular vesicles. Adult astrocytes on the other hand predominantly express TLR4 and, to a lesser extent, TLR3. On astrocytes TLR are expressed on the cell surface only.

Given the dominant function of TLR in controlling innate responses against invading pathogens in other cell types and tissues, TLR may play a crucial role in pathogen control also in the human CNS. Recently, we established a cell culture model for infecting human adult astrocytes with a common neurotropic human pathogen, viz. human herpesvirus-6 (HHV-6). HHV-6 is a ubiquitous DNA β-herpesvirus which persistently infects at least 95 % of the human population and can be detected in at least 40 % of normal healthy brains. HHV-6 infection in the adult CNS is usually silent and does not frequently trigger clinically or pathologically obvious inflammatory reactions. Like other herpesviruses, HHV-6 has developed several strategies to evade elimination by the host's immune system. These strategies include production of anti-inflammatory cytokine and chemokine mimics, or mimics of chemokine receptors to control the host innate immune response.

Especially in view of the ability of HHV-6 to maintain itself in the human adult CNS it is of interest to examine its interaction with the TLR signaling network of glial cells. In the present study real-time quantitative PCR was used to monitor expression levels of four TLR family members in astrocytes under the influence of HHV-6 infection over a period of 2 days. Also, we examined the effects of pro-inflammatory cytokines and monitored their production as well, as a read-out for possible TLR-mediated responses.

While the current preliminary study does not allow any far-reaching conclusions yet, the data suggest that both HHV-6 and pro-inflammatory cytokines trigger preferential induction of TLR3. Levels of mRNA encoding the abundant TLR4, or the much less abundant TLR1 and TLR2 were only moderately affected. The combination of HHV-6 infection and cytokine

treatment again triggered strong TLR3 induction but in this case, also TLR2 was induced at levels comparable to TLR3. As assessed by cDNA array analysis HHV-6 infection did not trigger any appreciable production of IL-1β, TNF-α, IL-6, type-I interferons or other mediators that are generally considered to be markers for TLR-mediated responses, including those for TLR3 and TLR4. Thus, in the time frame monitored in the present study experimental HHV-6 infection of human adult astrocytes does trigger elevated TLR expression but appears to fail to provide the ligands to activate these TLR.

Materials and methods

Donors

Astrocytes were obtained from post-mortem sub cortical white matter samples provided by the Netherlands Brain Bank after rapid autopsy. The Netherlands Brain Bank supplies post-mortem material from clinically well documented and neuropathologically confirmed cases. Autopsies are performed on donors with written informed consent from the donor or from direct next of kin. All procedures applied in the experiments were approved by the local ethics committee. Material from four control donors free from any clinical or pathological signs of neurodegenerative diseases was used. Additional donor characteristics are given below in Table 5.1.

Table 5.1. Donor characteristics

Donor nr	Donor#	Sex	Age	PM delay	Clinical history	Cause of death
1	00-022	female	83	7h 45m	non-demented control	acute myocard infarction
2	00-031	female	77	9h 15m	non-demented control	cardiac infarction
3	00-032	female	78	6h 30m	non-demented control	decompensatio cordis
4	01-104	female	77	5h 30m	non-demented control	lung metastases from a mammacarcinoma

PM = post mortem

Isolation and *in vitro* culture of human adult astrocytes

Human adult post-mortem astrocytes were obtained as previously described (5,6). Briefly, white matter samples were collected and meninges and visible blood vessels were removed before mincing the tissue into small cubes. The tissue fragments were incubated at 37°C for 20 min in 0.25 % trypsin (Sigma, St. Louis, MO) and 0.1 mg/mL bovine pancreatic DNAse I (Boehringer Mannheim, Germany). After digestion cell suspensions were

gently triturated, washed and taken into culture in medium containing 1:1 v/v DMEM : HAM-F10 supplemented with 10 % FCS (Biowhittaker, Verviers, Belgium) and pyruvate, glutamine and antibiotics. Astrocyte cultures were grown in 25 cm^2 culture flasks or 6-well plates (Costar, NY, USA) coated with poly-L-lysine (Sigma Chemical Co, St Louis, MO) until post-confluent monolayers were obtained at passage 4. The purity of the astrocyte cultures was verified by staining with rabbit anti-glial fibrillary acidic protein (GFAP; ZYMED, San Francisco, CA) as an astrocyte marker, murine anti-human CD68 (DAKO, Glostrup, Denmark) as a microglial marker, and murine anti-myelin basic protein (MBP; Boehringer Mannheim, Germany) as an oligodendrocyte marker. The astrocyte cultures were found to be at least 99% GFAP-positive. No cells were found to express CD68 or MBP.

Cytokine treatment of cultured human adult astrocytes

Previous studies have consistently indicated that changes in the gene expression profile of human astrocytes in response to pro-inflammatory cytokines are not generally rapid but accumulate over 72-h periods (5,7,8). Based on these findings astrocytes were stimulated with pro-inflammatory cytokines TNF-α, IL-1β and IFN-γ (PreproTech, Rocky Hill, NY) for periods of 2, 6, 24 and 48 h, respectively, in order to monitor TLR expression levels . Treatments were performed with each cytokine separately added to a final concentration of 500 U/mL, as well as with a mixture of the three cytokines at this concentration.

HHV-6 infection of human astrocytes

Astrocytes from donors 1, 2 and 4 were infected with HHV-6 as previously described in Chapter 3 and 4. Briefly, post-confluent astrocyte cultures were infected using a HSB-2 carrier cell line of which at least 75 % of the cells could be stained for the 105 kDa common antigen of HHV-6A and B (NCL-HHV-6; Novocastra, Newcastle, UK). Before adding this HHV-6-infected HSB-2 cell line to astrocytes, the carrier cells were washed and resuspended in fresh culture medium. HHV-6-infected HSB-2 cells and astrocytes were co-cultured for a period of 18 h, after which astrocytes were extensively washed to remove HSB-2 cells, fresh culture medium was added and the astrocytes were cultured for an additional 48 h.

Cells cultured and infected in chamber slides (Falcon, Becton Dickinson, NY) were washed with PBS and fixed in acetone supplemented with 0.03 % v/v H_2O_2 (Merck, Darmstadt, Germany) for 10 min. Chamber slides were air-dried and stained for GFAP (GFAP; ZYMED, San Francisco, CA) and NCL-HHV-6. Slides were embedded in vectashield mounting medium with

4'6-diamidino-2-phenylindole-2HCl (DAPI; Vector Laboratories, Burlingame, CA) for DNA staining. Astrocyte cultures obtained from donor 1 and 4 were infected once and astrocyte cultures obtained from donor 2 were infected twice using separately prepared post-confluent cultures.

Cytokine treatment of HHV-6-infected astrocytes
After HHV-6 infection as described above astrocytes were cultured for an additional 48 h in the presence of a mixture of TNF-α, IL-1β and IFN-γ, each at a concentration of 500 U/mL. Cytokine treatment was performed using astrocyte cultures from donor 1 and 4, and two separately prepared astrocyte cultures from donor 2.

RNA isolation and cDNA synthesis
To isolate RNA, cells were washed with PBS and RNA was isolated using RNAzol B (Campro Scientific, Veenendaal, The Netherlands) according to the manufacturer's instructions. Briefly, astrocytes were collected in RNAzol B and kept on ice. Chloroform was added to a final concentration of 10 % (Sigma-Aldrich Chemie, Steinheim, Germany), cells were shaken firmly and kept on ice for another 5 min and finally the sample was centrifuged for 15 min at 13,600 g at 4°C. RNA in the aqueous supernatant was precipitated with an equal volume of ice-cold isopropanol (Sigma-Aldrich chemie, Steinheim, Germany) and kept overnight at 4°C. RNA was collected by centrifugation for 30 min at 13,600 g at 4°C and the pellet was washed with 75 % ethanol and centrifuged again. After drying the pellet was dissolved in RNA-se free water.

Real-time polymerase chain reaction (RT-PCR)
Quantification of mRNA encoding β-actin, TLR1, TLR2, TLR3 and TLR4 was performed by quantitative real-time RT-PCR using an ABI PRISM® 7700 sequence detection system (Applied Biosystems). Data were analyzed using sequence detector version 1.7 software. cDNA was synthesized from 2.0 μg RNA using reverse transcription system (Promega, Madison, WI). Briefly the PCR reaction volume was 25 μL and contained a mixture of dNTPs, PCR buffer, Mg^{2+} (25 mM), beacon (1 μM), sense and anti-sense primer and Taq polymerase. Ten μL of cDNA (1:10 diluted in water) was added to the reaction mixture. Sequences for the fluorogenic molecular beacons and primers (Biolegio) used for β-actin, TLR1, TLR2, TLR3 and TLR4 transcription are listed in Table 5.2. PCR amplification reactions were conducted with an initial 5 min denaturation step at 95ºC coupled to a repeating cycle of 30 s at 95ºC, 40 s at 56ºC and 30 s at 72ºC for 40 cycles.

The amount of product was measured during the 20 to 40 s period at 56ºC, at this point the maximum amount of beacons is bound to the cDNA. To quantify the amount of the target gene we used known quantities of plasmids as standards carrying individual target cDNAs. As a positive control for the RT-PCR technique we used pooled cDNA from microglia cultures obtained from different donors (4). To correct for differences in initial cell numbers, as well as in the efficiency of mRNA isolation and cDNA synthesis all values were normalised for β-actin levels and TLR levels are expressed as the relative amount of transcripts.

The impact of HHV-6 infection on the production of pro-inflammatory cytokines and their receptors assessed by cDNA arrays

Analysis of the mRNA profile of HHV-6-infected astrocytes was performed by hybrid selection of radioactive labeled cDNA on high-density Clontech Atlas® arrays of membrane-bound cDNA probes, as described previously (8). Briefly, total cellular RNA was extracted and poly A^+ RNA was separated from total RNA. Radioactive cDNA probes were prepared by adding dNTP-mix, DTT, MMLV RT and [^{32}P]-dATP and incubating for 25 min at 50°C. Hybridization of the radioactive cDNA probes to the membrane was carried out overnight. Specifically bound radioactivity was analyzed and quantified by phosphor-imaging. The hybridization signal for each gene probe, present in duplicate on the array, was calculated as the mean of these duplicates, corrected for background intensity and quantified using software provided by the manufacturer. Relative hybridization signals were calculated by dividing the absolute signals by the mean signal for all 9 housekeeping reference genes on the corresponding array, and multiplying this ratio by 1,000.

Table 5.2. Primers en beacons for real-time quantitative PCR.

Human gene	Sense primer	Anti-sense primer	Product length	Beacon
β-actin	GGTCATCACCATTGGCAATGA	ACGTCACACTTCATGATGGAGTTG	123 bp	cgtgccGCACTCTTCCAGCCTTCCTTCCTGggcacg
TLR1	GAAGAAAGTGAATTTTTAGTTGATAGGTCA	ACAGTGATAAGATGTCAGAAGTCCAAAG	139 bp	cgtgccATCCACGTTCCTAAAGACCTATCCCAGAggcacg
TLR2	GAAATGTGAAAATCACCGATGAAAG	TCCACTTTACCTGGATCTATAACTCTGTC	160 bp	cgtgccTTTATGACTGTACCCTTAATGGAGTTggcacg
TLR3	CAGTACATCGAGTTCTTGGTTTCAAA	GAGAAATGTTCCCAGACCCAATC	115 bp	cgtgccCAGACAGACAGAACAGTTTGAATATGCAGCggcacg
TLR4	TAAAGAATTTAGAAGAAGGGGTGCC	CAACAATCACCTTTCGGCTTTTA	129 bp	cgtgccGAGACTTTATTCCCGGTGTGGCCAggcacg

bp = base pairs
all sequences are given from 5’ to 3’ end

Results

The impact of TNF-α, IL-1β or IFN-γ on TLR expression in astrocytes

First, we examined the effects of pro-inflammatory cytokines on expression levels of TLR in astrocytes to establish a suitable time frame to monitor the possible effects of HHV-6 infection. Traditional pro-inflammatory mediators TNF-α, IL-1β, IFN-γ and a mixture of these three cytokines together were used to stimulate cultured astrocytes and TLR expression levels were monitored by quantitative real-time PCR over a 48-h period, using expression levels of β-actine encoding mRNA as a reference. The present analysis was focused on TLR1 through TLR4 since expression levels of TLR5 through TLR10 in cultured human astrocytes are very low and remain so in the presence of pro-inflammatory cytokines (Bsibsi et al., unpublished observations). Astrocytes derived from four different donors were used to evaluate possible donor-to-donor variations in the baseline values as well in patterns of responsiveness.

Expression levels of TLR1 (Figure 5.1) were found to be relatively low in all four donors. Real time PCR analysis indicated TLR1 transcripts in astrocytes at the start of the culture at levels 10^5 to 10^6 times lower than those encoding β-actin. Donor-to-donor variations in these levels were significant and expression levels of TLR1 differed in individual cultures by as much as a factor of 10. Upon treatment with either individual cytokines or a mixture of cytokines, levels of TLR1 transcripts markedly increased only in one culture but not in the other three. It should be noted, however, that the increase was observed for the culture with the lowest baseline expression of TLR1 and the 5- to 10-fold cytokine-induced increase only led to the low TLR1 levels already observed in the other cultures at baseline.

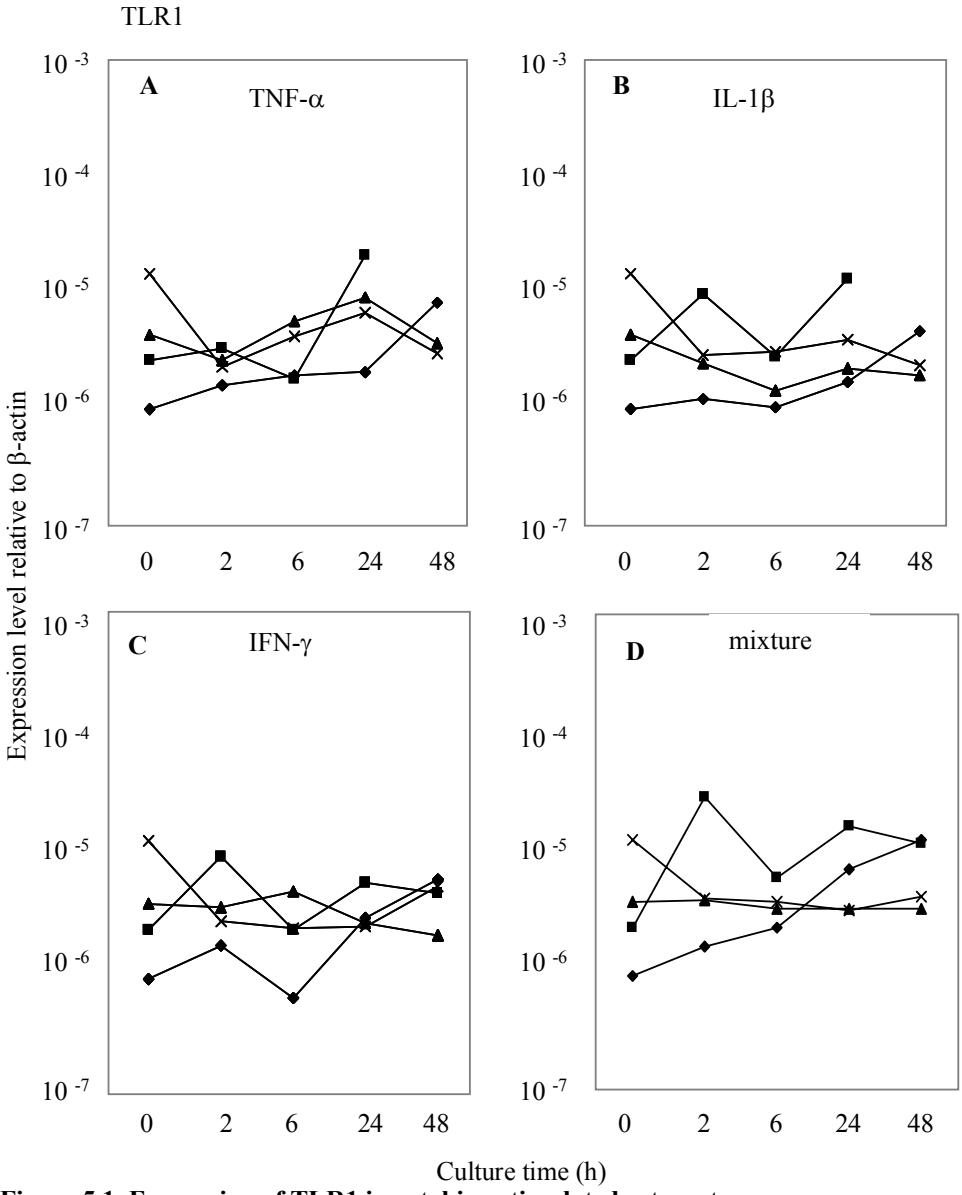

Figure 5.1. Expression of TLR1 in cytokine-stimulated astrocytes
Cultured human adult astrocytes were stimulated with either TNF-α (A), IL-1β (B) or IFN-γ (C) alone, or with a mixture of all three cytokines together (D). At the start of the experiment and after 2, 6, 24 and 48 h, levels of transcripts encoding TLR1 were determined by real-time PCR and compared to levels of transcripts encoding β-actin as an internal reference.

The levels of TLR2-encoding transcripts at baseline were very similar to TLR1-encoding transcripts (Figure 5.2). In this case, treatment with either TNF-α alone or the combination of TNF-α, IL-1β and IFN-γ together led to an increase of TLR2 levels up to 40-fold in three out of four cultures over a 48-h culture period. No such stimulatory effect was seen after treatment with IL-1β or IFN-γ alone, in which case none of the transcripts increased more than 4-fold. The amounts of TLR3 transcripts in cultures of untreated astrocytes were found to be around 10^{-5} relative to those of β actin, about five to ten times higher than those of TLR1 and TLR2 (Figure 5.3). Treatment with either TNF-α alone or the combination of TNF-α, IL-1β and IFN-γ together led to a marked (5- to 100-fold) increase of TLR3 levels in three out of four cultures. The one astrocyte culture that did not respond to cytokine treatment was the same culture that also failed to respond when monitored for TLR2. Effects of either IL-1β or IFN-γ alone were more modest with markedly (up to 40-fold) increased levels of TLR3 in only two cultures. Levels of TLR4-encoding transcripts in the astrocytes cultures were about 10 times higher as compared to TLR3-encoding transcripts, consistent with previous data illustrating that mRNA expression levels of TLR4 are dominant in cultured astrocytes. Effects of pro-inflammatory cytokines on these TLR4 expression levels were marginal in all cases, i.e. none of the cytokines nor the mixture of cytokines induced levels of TLR to change more than 7-fold (Figure 5.4).

The above data therefore indicate that especially TNF-α exerts a stimulatory effect on expression levels of mRNA encoding TLR3 and –albeit to a somewhat lesser extent- TLR2 but not TLR1 or TLR4. In general, elevated levels of TLR2- and TLR3-encoding transcripts were readily detectable after 24 and 48 h but not in the first 6 h.

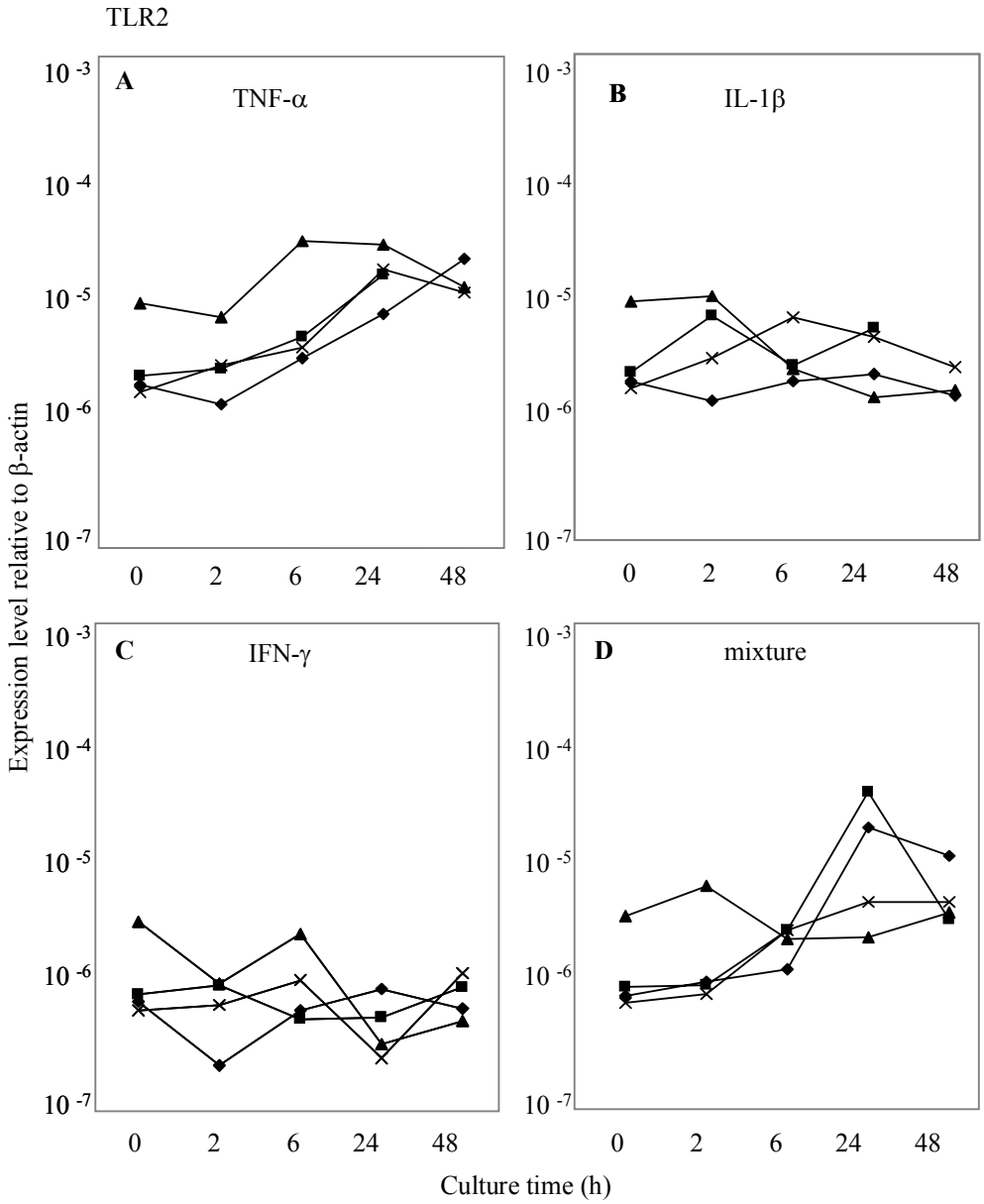

Figure. 5.2. Expression of TLR2 in cytokine-stimulated astrocytes

Cultured human adult astrocytes were stimulated with either TNF-α (A), IL-1β (B) or IFN-γ (C) alone, or with a mixture of all three cytokines together (D). At the start of the experiment and after 2, 6, 24 and 48 h, levels of transcripts encoding TLR2 were determined by real-time PCR and compared to levels of transcripts encoding β-actin as an internal reference.

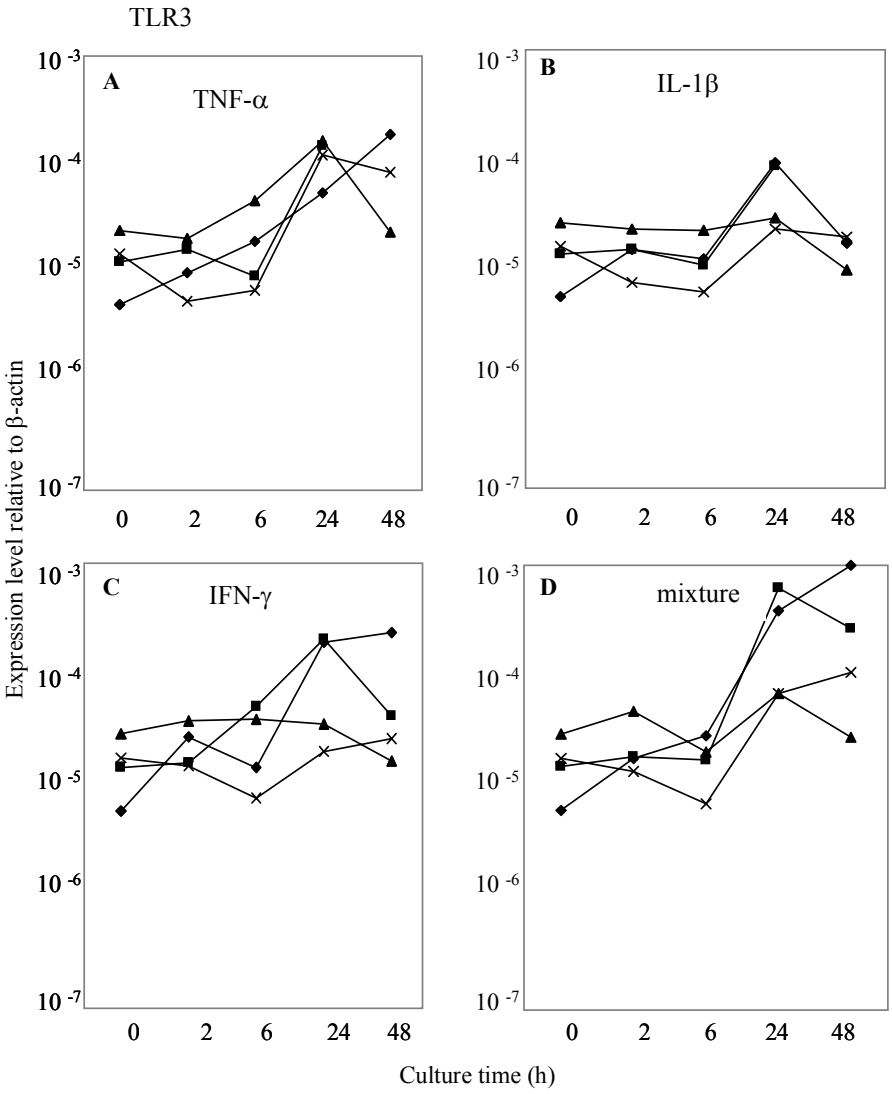

Figure 5.3. Expression of TLR3 in cytokine-stimulated astrocytes
Cultured human adult astrocytes were stimulated with either TNF-α (A), IL-1β (B) or IFN-γ (C) alone, or with a mixture of all three cytokines together (D). At the start of the experiment and after 2, 6, 24 and 48 h, levels of transcripts encoding TLR3 were determined by real-time PCR and compared to levels of transcripts encoding β-actin as an internal reference.

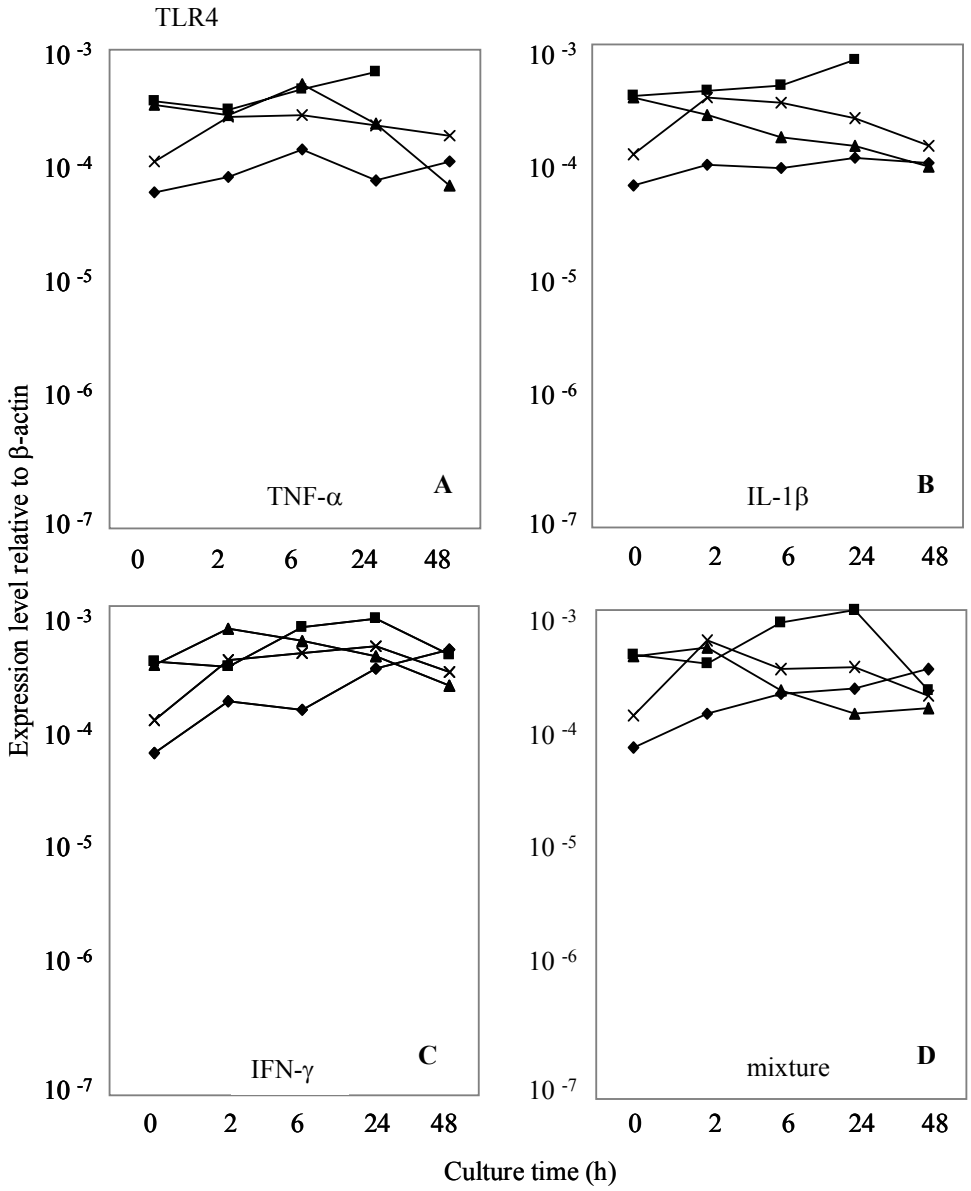

Figure 5.4. Expression of TLR4 in cytokine-stimulated astrocytes
Cultured human adult astrocytes were stimulated with either TNF-α (A), IL-1β (B) or IFN-γ (C) alone, or with a mixture of all three cytokines together (D). At the start of the experiment and after 2, 6, 24 and 48 h, levels of transcripts encoding TLR4 were determined by real-time PCR and compared to levels of transcripts encoding β-actin as an internal reference.

The impact of HHV-6 infection on TLR expression in astrocytes

The above data indicate that effects of cytokines on expression levels of TLR were apparent after 24 or 48 h but not after shorter periods of 2 to 6 h. This relatively slow response of astrocytes at the level of mRNA is fully consistent with previous observations of astrocyte cytokine-encoding mRNA responses both in our own studies and in those reported by others. Together, the data led us to select a period of 48 h to allow the effects of experimental HHV-6 infection of astrocytes to develop. In examining the effect of HHV-6 on expression levels of TLR-encoding transcripts we also included an experimental condition in which HHV-6 infection was combined with the addition of a mixture of TNF-α, IL1β and IFN-γ. This mixed condition of HHV-6 infection and pro-inflammatory cytokine treatment was chosen since previous studies had indicated that HHV-6 may exert very different effects on astrocytes under normal *versus* pro-inflammatory culturing conditions (8).

To investigate the impact of HHV-6 on TLR expression, astrocytes were supplied in culture with an HHV-6-infected carrier T-cell line (HSB-2). After an overnight co-culture period infected HSB-2 cells were removed and astrocytes were cultured for an additional 48 h. Under these conditions, between 30 % and 35 % of all astrocytes were infected as revealed by immunocytochemical staining for the 105 kDa antigen of HHV-6 in the nucleus of infected cells. Also marked cytopathic effects (CPE) including enlargement of nuclei, cell swelling and syncytium formation were apparent in about one third of the cells. After 48 h expression levels of TLR1-4 were measured using real-time PCR, again using β-actin-encoding transcripts as an internal reference.

First of all, the results revealed marked donor-to-donor variations in the effects of HHV-6 infection as shown in Figure 5.5.B. As already observed after cytokine treatment, the results of which for the 48-h observation period are represented again in Figure 5.5.A as a reference, the response levels found in individual astrocyte cultures sometimes show marked differences making it difficult to draw any far-reaching conclusions. Yet, three out of four cultures showed a 10- to 200-fold increase in TLR3-encoding transcripts while TLR1, 2 or 4 transcripts were generally affected less than 10-fold by HHV-6. None of the cultures showed any downregulation of TLR-encoding transcripts as the result of HHV-6 infection. These data suggest that over the 48-h period following infection, HHV-6 tends to

induce expression of TLR, with TLR3 affected more strongly than the other TLR.

We also monitored the effect of a combination of TNF-α, IL-1β and IFN-γ on TLR expression in HHV-6-infected astrocytes. After 48 h of infection, no change was observed in the percentage of HHV-6-infected astrocytes in culture under the influence of the cytokine mixture and again, about one third of the cells were infected. Figure 5.5.C shows the levels of TLR-encoding transcripts in HHV-6-infected astrocytes treated with the cytokine mixture, as compared to uninfected and untreated astrocytes. Overall, the combined effects of infection and cytokine treatment on expression levels of TLR1, TLR3 and TLR4 were very similar to those observed in infected astrocytes only. Again, TLR3 levels were markedly induced between 5- and 100-fold for all cultures and also for TLR1 and TLR4, the more modest levels of induction (up to maximally 20-fold) were very similar to those found in the absence of the combined cytokine stimulus. Yet, the effects on TLR-2-encoding transcripts were clearly influenced by the cytokines. While HHV-6 infection alone led to only very modest effects (10-fold induction in only one of the four cultures) the combination of HHV-6 infection with TNF-α, IL-1β and IFN-γ resulted in 10- to 100-fold induction of TLR2 in three out of the four cultures. Overall, the induction levels of TLR2 under these conditions were very similar to those for TLR3.

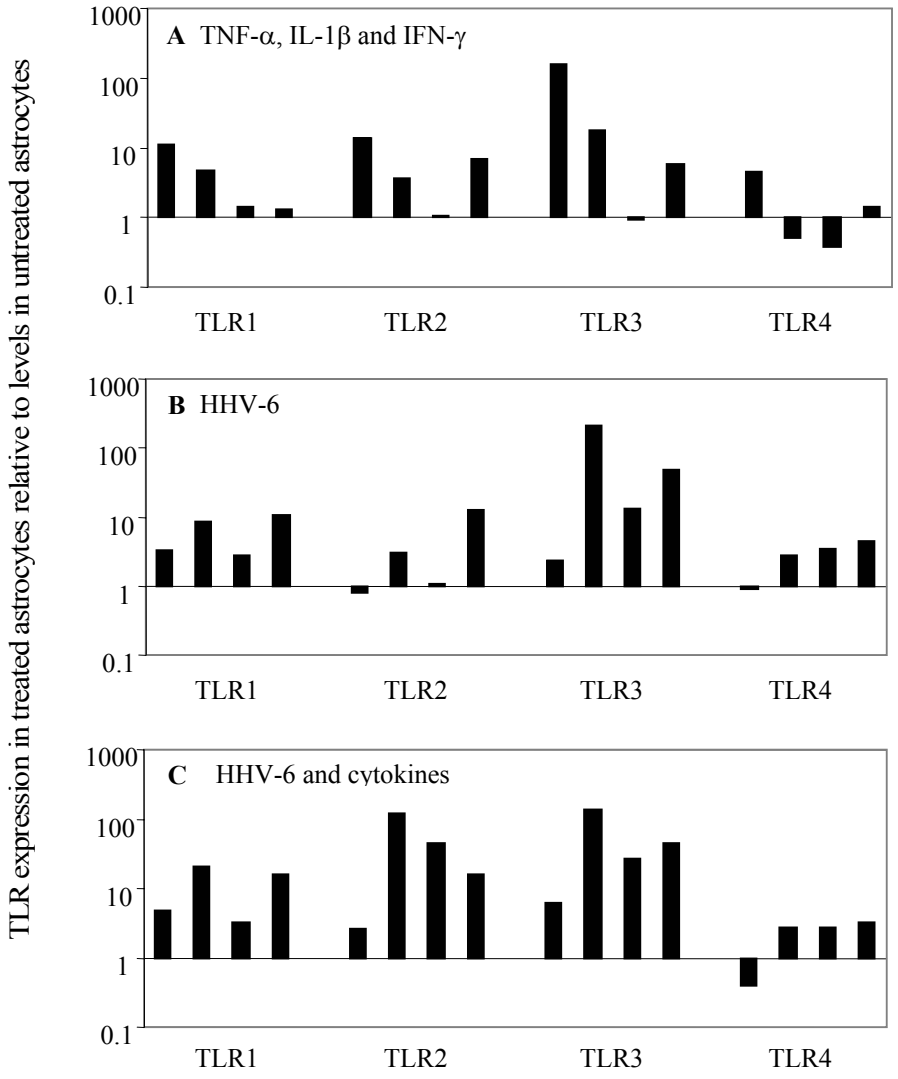

Figure 5.5. Expression levels of TLR1-4 in astrocytes under the influence of HHV-6 infection and cytokines

Cultured human astrocytes were stimulated with a mixture of TNF-α, IL-1β and IFN-γ (A), infected with HHV-6 (B) or infected with virus in the presence of cytokines (C). After 48 h levels of transcripts encoding TLR1-4 were determined by real-time PCR using transcripts encoding β-actin as an internal reference, and expressed relative to the levels found in an untreated control culture of astrocytes from the same source kept for 48 h under identical conditions. For each condition four separate astrocyte cultures from different donors were used to generate data.

The impact of HHV-6 infection on the production of pro-inflammatory cytokines

Next, we examined HHV-6-infected astrocytes for levels of expression of a range of mRNAs encoding cytokines, chemokines and growth factors. Levels of these mediators were evaluated for two reasons. First, TLR expression levels in HHV-6 infected astrocyte cultures could be influenced indirectly by cytokines or other mediators secreted into the culture medium by infected astrocytes. Figures 5.1-5.4 illustrate that soluble mediators notably including TNF-α can in fact influence TLR expression levels. Secondly, by determining expression levels of the pro-inflammatory cytokines and chemokines that are typically triggered by TLR ligation, including for example TNF-α, IL-6, IL-1β or type-I interferons, such ligation by any possible TLR ligand associated with HHV-6 infection may be revealed.

As reportedly previously, cDNA array analysis of expression of 268 cytokines, chemokines, growth factors and their receptors in HHV-6 infected astrocytes revealed marked induction only of a few genes. These genes, as listed in Table 4.1 of Chapter 4, include none of the traditional TLR activation markers as reported in literature with the only exception perhaps of CCL5 (RANTES) that is known as one of the products that could –among other factors- be triggered by TLR engagement. CCL5 expression is elevated 2- to 4-fold in two out of three cultures. Yet, when a focus is chosen on cytokines and chemokines that have been repeatedly found as TLR-induced gene products in a variety of cell types, none of these were found to be consistently induced by HHV-6 (Table 5.3) over a 48-h period. Especially noteworthy is the lack of any induction of IFN-β, a type-I interferon that is typically induced by TLR3. Since TLR3 is induced in HHV-6-infected astrocytes more strongly than the others, ligation of this particular TLR could well have been reflected by an increase in IFN-β, but it is not.

Table 5.3. Expression of pro-inflammatory cytokines and chemokines in HHV-6-infected astrocytes.

	untreated Signal intensity			HHV-6-infected Signal intensity			Ratio			
Gene	Exp 1	Exp 2	Exp 3	Exp 1	Exp 2	Exp 3	Exp 1	Exp 2	Exp 3	**Gene code**
tumor necrosis factor alpha (TNF-α)	118	330	71	178	60	307	0.18	1.52	4.35	X01394
interleukin-1 beta (IL-1β)	371	465	375	524	372	416	0.80	1.41	1.11	K02770
interferon-alpha2 (IFN-α)	108	73	93	66	<10	20	0.14	0.61	0.21	J00209
interferon-beta (IFN-β)	190	382	356	155	140	310	0.37	0.82	0.87	M28622
interferon gamma (IFN-γ)	286	53	139	163	12	35	0.23	0.57	0.25	X01992
interleukin-6 (IL-6)	342	163	429	545	348	491	2.13	1.59	1.14	X04602
interleukin-12 alpha subunit (IL-12α)	83	<10	78	37	<10	77	0.99	1.00	0.99	M65291
interleukin-12 beta subunit (IL-12β)	205	298	346	139	157	394	0.68	0.53	1.14	M65290
interleukin-17 (IL-17)	123	<10	43	92	<10	20	0.75	1.00	0.47	U32659
CCL2 (MCP-1)	722	580	788	975	736	983	1.35	1.27	1.25	M24545
CCL3 (MIP1-α)	96	59	91	84	<10	133	0.88	0.17	1.46	M23452
CCL4 (MIP1-β)	70	<10	47	76	<10	35	1.09	1.00	0.73	J04130
CXCL6 (GCP-2)	120	<10	27	89	44	62	0.74	4.40	2.30	X78686

Discussion

In the present study we investigated the impact of pro-inflammatory cytokines and HHV-6 infection on TLR expression in cultured human adult astrocytes. We focussed on the expression of TLR1, TLR2, TLR3 and TLR4 since these family members are expressed at detectable levels in cultured human adult astrocytes while TLR5 to TLR10 are not detectable, also not after treatment with pro-inflammatory cytokines (Bsibsi et al., unpublished observations). As a first approach, the present study was based on an analysis of astrocyte cultures from four different donors. Since marked donor-to-donor variations in levels of TLR-encoding transcripts typify results in most cases, a more extended analysis will be required to allow for firm conclusions. Also, validation of expression data at the level of protein expression will be useful to support the current data. Finally, it should be noted that experimental infection of astrocyte cultures leads to infection of about one third of the cells, which will probably obscure more subtle effects of infection that can only be revealed at higher percentages of infection. Bearing these caveats in mind, however, the data collected in the present study do allow some trends to be already identified.

The levels of transcripts encoding TLR1-4 in normal adult astrocyte cultures as observed in the present study are consistent with a previous report on TLR expression in astrocytes (4) in demonstrating dominant expression of TLR4. TLR4 is expressed at levels that about ten times higher than those for TLR3, the second dominant TLR in cultured astrocytes. TLR1 and TLR2 are expressed at only very low levels. In response to TNF-α, TLR3, and to a lesser extent TLR2, are induced in astrocytes over a 24- to 48-h period. Other pro-inflammatory mediators including IL-1β and IFN-γ appear to have much less effect. At earlier points in time following TNF-α treatment, levels of TLR-encoding transcripts did not change to any significant level, suggesting that the cytokine-induced effects of expression of TLR are relatively slow and follow the response course previously documented at the level of a large variety of cytokine and chemokine mRNAs. The apparently preferential induction of TLR3 by pro-inflammatory cytokines is remarkable and deserves follow-up experiments using more donors and analyzing TLR expression also at the protein level.

Also after HHV-6 infection of astrocyte cultures, TLR3 induction appears to be the dominant effect. In three out of the four cultures, TLR3 was induced by a factor of between 10 and 200, levels of induction that were not attained by any other TLR. Bearing in mind that only one third of the cells are actually infected with HHV-6, and that the effect is most likely not an

indirect one that is mediated by cytokines or chemokines secreted by infected cells (see below), this level of response is very strong. Clearly, induction of TLR and especially of TLR3 at the level of mRNA should be verified by protein analysis including immunocytochemical staining. This should also clarify whether enhanced TLR expression is indeed confined to infected cells, or also occurs on uninfected cells. As stated above, it is unlikely that TLR induction by HHV-6 infection of the astrocyte cultures is mediated indirectly by cytokines or other mediators secreted by infected cells. HHV-6-infected astrocyte cultures hardly produce any soluble pro-inflammatory mediators such as TNF-α that could exert such an effect (see Figure 4.1 and Table 4.1).

The lack of such mediators being triggered in astrocytes by HHV-6 infection has another interesting implication. Several mediators including TNF-α, IL-1β and IL-6 are consistent products of TLR activation in a variety of cell types, and traditional products of NF-κB-mediated signaling that is activated as a common response to all TLR. In the case of TLR3, also type-I interferons including IFN-α and IFN-β are generally activated. The fact that over the 48 h-monitoring period mRNA for none of these mediators are found at levels that are any higher than in control cultures strongly suggests that activation of TLR-mediated signaling does not occur in HHV-6-infected astrocyte cultures. In turn, this suggests that while HHV-6 infection does apparently lead to elevated levels of expression of TLR, infection does not provide the ligands such as dsRNA to activate these TLR in the time frame of the current study. This lack of TLR activation may be at least partly explained by the fact that TLR are only expressed on the surface of astrocytes whereas the virus is intracellular and no evidence has so far been found for viral particles to be relased from infected astrocytes in culture.

Finally, the present study suggests that TLR induction in HHV-6-infected astrocyte cultures is altered by the addition of pro-inflammatory cytokines in one selective feature, viz. induction of TLR2. While induction levels of TLR1, TLR3 and TLR4 remain essentially the same in HHV-6 infected astrocyte cultures irrespective of the presence of a mixture of TNF-α, IL1β and IFN-γ, induction levels of TLR2 are markedly increased by these cytokines. No downregulation of TLR expression was observed under these conditions. Again, this phenomenon deserves to be examined more closely also at the protein level. It is not immediately obvious which relationship HHV-6 could bear to TLR2, especially since TLR2 is known to respond to quite a variety of different ligands including lipopeptides, lipoproteins, zymosan, peptideoglycan and heat-shock proteins.

While representing only a first step towards understanding the role of TLR in virus infection in the human CNS, the present study does represent the first documentation of the effects of a viral infection on TLR in human adult astrocytes. Data on the effects of viruses on TLR in other cell types are beginning to emerge and they are revealing complex and sometimes opposing effects. Infection of macrophages with influenza A and Sendai viruses enhances expression of TLR1, TLR2 and TLR3 (9). Wild-type measles virus, human cytomegalovirus (HCMV) and herpes simplex virus 1 (HSV-1) activate monocytes via TLR2 and trigger inflammatory cytokine production (10,11,12). TLR4 plays a role in anti respiratory syncytial virus (RSV) responses (13). Vaccina virus (VV) on the other hand inhibits multiple TLR pathways to NF-κB and thus suppresses the TLR-mediated host immune response (14). Very often, TLR3 is associated with the response against viruses since the predominant response to TLR3 ligand is production of type-I interferons, which is generally considered as a typical anti-viral response. Also, TLR3 is known to be activated by double-stranded RNA which is a replication intermediate for several viruses. Nevertheless, Edelmann and co-workers recently showed that the susceptibility to reovirus, lymphocytic choriomeningitis virus (LCMV), vesicular stomatitis virus (VSV) and murine cytomegalovirus (MCMV) was unaffected in TLR3-/- mice (15). On the other hand, wild type mice infected peripherally with West Nile virus show enhanced BBB breakdown and elevated levels of brain infection as compared to TLR3-/- mice (16).
These observations are only the first sets of data that will ultimately be required to gain a full understanding of the interaction between viruses and TLR. While requiring confirmation and extension, our current data appear to point to TLR3 and TLR2 as being of prime relevance to the interaction between HHV-6 and human adult astrocytes, also under inflammatory conditions. While HHV-6 most likely leads to marked induction of TLR on astrocytes, it appears to fail in directly activating these TLR. This suggests that TLR on astrocytes may play alternate roles in the host response to infection in the human CNS, different from directly engaging pathogen-derived components.

Acknowledgements
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CHAPTER 6

Cultured human adult microglia from different donors display stable cytokine, chemokine and growth factor gene profiles but respond differently to a pro-inflammatory stimulus

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Abstract
Brain microglia are highly responsive cells in the central nervous system that exert key functions in host defense as well as in neuroprotection and regeneration. In this study the gene expression profiles for 268 cytokines, chemokines, growth factors and their receptors were examined in cultures of purified human adult microglia, using cDNA array profiling.
Microglia from nine different donors were compared, also following challenge of such microglia with the pro-inflammatory cytokines TNF-α and IFN-γ. A stable pattern was observed of genes abundantly expressed in the different cultures under standard conditions. Genes abundantly expressed in all microglia cultures include CCL2 (MCP-1), thymosin beta-10, migration inhibitory factor-related protein 8 (MRP8), MRP14, corticotropin releasing factor receptor 1 and endothelin 2. Abundant gene products novel to microglia were neuromodulin (GAP43) and Flt3 ligand. Yet, treatment with TNF-α and IFN-γ led to widely different response profiles among the different cultures.
These data show a surprising level of heterogeneity among human adult microglia cultures in their response to a pro-inflammatory stimulus despite the standardized methodology to examine this response.

Introduction

Microglial cells are derived from $CD45^+$ bone marrow precursors of the myeloid lineage, which also give rise to macrophages, dendritic cells and granulocytes (1,2,3). Microglia compose approximately 5 to 20 % of all cells in the central nervous system (CNS) (4,5). They are the gatekeepers of the brain and function as sensor cells that are continuously in surveillance of changes or insults in the CNS. Microglia respond to signals from stressed or damaged cells as well as to invading pathogens. The adult brain has at least two major subsets of resident phagocytic cells. These are resting microglia in the parenchyma and perivascular macrophages located in the basal lamina of brain vessels and in the choroid plexus (6). The replacement rate of perivascular macrophages by blood-derived mononuclear cells is relatively fast whereas that of ramified microglia in the parenchyma is very slow. In contrast, a high turnover rate has been described for microglia in damaged or injured brains (7,8).

Upon activation by brain damage or injury microglia loose their normal ramified shape and acquire a macrophage/monocyte-like rounded morphology (9). Under such conditions, microglia proliferate and migrate through the brain. Chemokines including CCL2 (MCP-1), CCL3 (MIP-1α) and CCL4 (MIP-1β) appear to play important roles in this response (10, 11,12). Activated microglia participate in the signaling network in the CNS during inflammation and consequently, they are involved in the pathogenesis and/or regulation of a variety of neurodegenerative disorders including multiple sclerosis (MS) (13), Alzheimer's disease (AD) (14), Parkinson's disease (15) and HIV encephalitis (16). Activated microglia increase their phagocytic activity and also express higher levels of major histocompatibility complex molecules (MHC) and co-stimulatory molecules as compared to resting, ramified microglia. Consequently, they acquire antigen-presenting functions (13). The activation of microglia is likely to involve a stepwise differentiation program similar to that governing dendritic cell maturation (17). Details of this differentiation program, however, remain to be clarified. Differentiation, migration and other microglia functions thus appear to be governed by complex signaling networks involving a variety of mediators including cytokines, chemokines and growth factors.

As a first approach to understanding such networks, *in vitro* studies of gene profile expression in microglia are useful. Whether or not a representative and reproducible gene profile can be obtained of human microglia cultured from different donors is a question to be answered for such an approach.

Isolating and culturing microglia in itself will already trigger some activation, given the innate responsiveness of microglia. An important question is therefore whether cultured and partly activated microglia reliably reflect the behavior of microglia *in vivo*, and respond in ways that are similar to microglia reactions in an intact CNS to trauma, infection or inflammation.
To address this issue, we examined by cDNA array profiling the expression pattern of 268 cytokines, chemokines, growth factors and their receptors in human adult microglia isolated from post-mortem brain samples of nine different donors. In a previous study we have demonstrated in detail the reliability and reproducibility of such a comprehensive read-out system, applying it to cultured human adult astrocytes (18). Microglia were examined under standard culture conditions in the presence of GM-CSF, and after treatment with either TNF-α or IFN-γ. When isolated and cultured under standard conditions microglia from all donors display a very similar pattern of gene expression that is in line with mediators produced in (activated) microglia *in vivo*. Intriguingly, however, pro-inflammatory cytokines triggered markedly different responses in different microglia cultures. These findings suggest that human adult microglia from different sources and/or regions of the CNS may have an innate propensity to respond in different ways to a pro-inflammatory insult.

Materials and methods

Donors

Microglia were obtained from post-mortem sub cortical white matter provided by the Netherlands brain bank after rapid autopsy. The Netherlands Brain Bank supplies post-mortem material from clinically well documented and neuropathologically confirmed cases. Autopsies are performed on donors with written informed consent from the donor or from direct next of kin. All procedures applied in the experiments described in our paper have been approved by the local ethics committee of the free University of Amsterdam. Material from nine different donors was used including two control donors (without any neurological disorder), four donors with Alzheimer's disease (AD), one donor with Multiple sclerosis (MS), one donor with non-Alzheimer dementia and one donor with dementia with senile involutive cortical changes and Lewy bodies. Additional donor characteristics are given in Table 6.1.

Table 6.1. Donor characteristics

Donor nr	Donor#	Sex	Age	PM delay[a]	Clinical history[b]	Cause of death
1	01-002	female	83	3h 30m	Dementia with s.i.c.c./L.b.v.	cachexia, dehydration
2	01-006	female	91	5h 45m	non-demented control	sudden death, suspected myocard infarction
3	01-010	female	84	4h 15m	AD	cachexia, dehydration
4	01-013	female	68	4h 51m	AD	dehydration
5	01-027	female	50	14h 35m	non-alzheimer dementia	suicide
6	01-033	male	75	6h 20m	non demented control	dehydration, pneumonia
7	01-042	female	71	6h 45m	AD	dehydration
8	03-052	male	53	5h 30m	MS	respiratory insufficience
9	03-057	female	68	3h 50m	AD	pneumonia, dehydration

[a] PM = post-mortem
[b] s.i.c.c. : senile involutive cortical changes; L.b.v.: Lewy bodies variant no Alzheimer's disease or Parkinson's disease ; AD: Alzheimer's disease; MS: multiple sclerosis

Isolation and *in vitro* culture of human adult microglia

Human adult post-mortem microglia were obtained as previously described (19,20). Briefly, sub cortical white matter samples were collected and meninges and visible blood vessels were removed before mincing the tissue into small cubes. The tissue fragments were incubated for 20 min at 37°C in 0.25 % trypsin (Sigma, St. Louis, MO) and 0.1 mg/mL bovine pancreatic DNAse I (Boehringer Mannheim, Germany). After digestion, cell suspensions were gently triturated and washed with culture medium containing DMEM/HAM-F10 supplemented with 10 % fetal calf serum (FCS; Biowhittaker, Verviers, Belgium) and antibiotics. After centrifuging for 10 min at 470 g at 20°C the cell suspensions were passed through a 100-μm nylon filter (Falcon, Franklin Lakes, NJ) and myelin was removed by Percoll gradient centrifugation for 30 min at 1,400 g at 20°C. To lyse erythrocytes cells were resuspended in a solution containing 155 mM NH_4Cl, 1 mM $KHCO_3$ and 0.2 % BSA and kept on ice for 20 min. After centrifuging for 10 min at 470 g at 20°C the cell pellet was washed once with culture medium and microglia were plated in 25 cm^2 culture flasks or 6-well plates (Costar, NY, USA). Recombinant human granulocyte-macrophage colony stimulating factor (rhGM-CSF; PeproTech Inc, Rocky Hill, NJ) promoting *in vitro* proliferation of microglia (21,22) was added at a concentration of 20 ng/mL in fresh culture medium every 3 days. The purity of the microglia cultures was verified by staining with murine monoclonal anti-human CD68 (DAKO, Glostrup, Denmark) as a microglial marker (23), rabbit polyclonal anti-glial fibrillary acidic protein (GFAP; ZYMED, San Francisco, CA) as an astrocyte marker, and murine monoclonal anti-myelin basic protein (MBP; Boehringer Mannheim, Germany) as an oligodendrocyte marker. The microglia cultures were found to be essentially 100 % CD68-positive. No cells were found to express GFAP or MBP. Microglia were cultured 10 to 14 days and additionally treated for 48 h with 500 U/mL of either recombinant human TNF-α or IFN-γ (PreproTech Inc, Rocky Hill, NJ). Microglia from donors 1, 2, 6, 7 and 8 were treated with TNF-α and microglia from donors 4, 5 and 9 were treated with IFN-γ.

RNA isolation

Culture medium was removed and cells were washed with PBS. RNA was isolated using RNAzol B (Campro scientific, Veenendaal, The Netherlands) according to the manufacturer's instructions. Briefly, microglia were collected in RNAzol B and kept on ice. Chloroform was added to a final

concentration of 10 % (Sigma-Aldrich chemie, Steinheim, Germany) and cells were shaken firmly and kept on ice for another 5 min and centrifuged for 15 min 14,000 g at 4°C. The aqueous solution was transferred to a new tube and an equal volume of ice-cold isopropanol (Sigma-Aldrich chemie, Steinheim, Germany) was added, precipitation of RNA was carried out overnight at 4°C. After precipitation the samples were centrifuged for 30 min 14,000 g at 4°C and the pellet was washed with 75 % ethanol and centrifuged again. After drying the pellet was dissolved in RNA-se free water.

mRNA profiling using cDNA arrays

Analysis of the mRNA profile of microglia was performed by hybrid selection of radioactive labeled cDNA on high-density Clontech Atlas® arrays of membrane-bound cDNA probes, as previously described (18). Briefly, total cellular RNA was extracted from the microglia and poly A^+ RNA was separated from total RNA. Radioactive cDNA probes were prepared by adding dNTP-mix, DTT, MMLV RT and [^{32}P]-dATP and incubating for 25 minutes at 50°C. Hybridization of the radioactive cDNA probes to the membrane was carried out overnight. Specifically bound radioactivity was analyzed and quantitated by phosphor-imaging. The hybridization signal for each gene probe, present in duplicate on the array, was calculated as the mean of these duplicates, corrected for background intensity and quantified using software provided by the manufacturer. Relative hybridization signals were calculated by dividing the absolute signals by the mean signal for all 9 housekeeping reference genes on the corresponding array, and multiplying this ratio by 1,000. Statistical analysis was performed using Pearson correlation analysis to compare gene profiles from different microglia cultures before and after cytokine treatment.

Results

Gene profiles of human microglia from different donors

Human adult CD68-positive primary microglia in culture provided with GM-CSF developed small cell bodies and a bi-polar morphology over periods of 1 to 2 weeks (Figure 6.1).

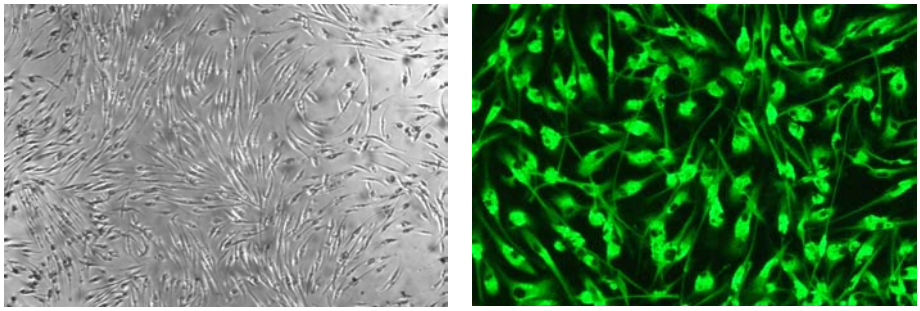

Figure 6.1. Human adult microglia in culture

Microglia were isolated from sub cortical white matter and cultured for 2 weeks in the presence of GM-CSF as described in materials and methods. Microglia displayed a uniform morphology typified by small cell bodies and a bipolar shape (A; phase contrast microscopy, magnification 10x). Microglia cultures were found to be essentially 100 % pure as verified by staining for CD68 (B; magnification 20x).

In this period, microglia did not show the highly ramified morphology that typifies resting microglia in an intact CNS but a bi-polar shape, reflecting a distinct state of activation induced by isolating and culturing the cells. Gene expression profiles were recorded for cultures of microglia derived from nine different donors using cDNA arrays that allow monitoring of 268 genes encoding cytokines, chemokines, growth factors and their receptors.
The profiles of the twenty most abundantly expressed genes encoding cytokines, chemokines, growth factors and their receptors were very similar for the microglia cultures obtained from the different donors. Table 6.2 lists those profiles along with standard deviations and coefficients of variation in the nine separate analyses. Among the most abundantly expressed genes are several chemokines such as CCL2, CCL3 and CXCL8. Also the migration inhibitory factor-related proteins (MRP) 8 and MRP14 are abundantly expressed, which are known to be expressed at elevated levels in microglia in traumatic brain injury or during cerebral malaria (24,25). Together with high expression of chemokines, expression of these MRP markers therefore confirm that cultured microglia are different from fully resting microglia, consistent with their altered morphology. In addition to the above gene products, three family members of bone morphogenetic proteins (BMP) including BMP4, BMP2A and BMP2B are highly expressed. These

mediators are known to be involved in different stages of neural development and exert functions in neuroprotection (26). In fact, several other mediators expressed at high levels in microglia also appear to have a predominant function in neuroprotection and regeneration. This for example also applies to pleiotrophin and its closely related gene HBNF-1 that promote growth and neurite extension (27), endothelin-2 (28,29,30), nerve growth factor receptor and neuromodulin (or GAP-43) (31). Neuromodulin has not been documented before in human microglia, neither has the currently documented expression of ribonuclease angiogenin inhibitor and Flt3-ligand. Especially the latter novel microglia product is of interest since Flt3 and its ligand have recently gained increasing attention as key mediators in dendritic cell development (32) and our current data therefore suggest that a similar pathway may control also microglia development.

To validate the results of the gene profiling analysis, we compared our data to the set of microglial gene products that have been documented previously by others (Table 6.3). To the extent that they were represented on the array, all products previously found by others in human microglia *in vivo*, or in cultured human microglia, using varying techniques including immunohistochemistry, PCR or ELISA were also detected by the current analysis. In all cases, expression levels were at least 10 % of the mean levels found for the set of house-keeping genes that served as a reference. This confirms that cultured microglia in their partially activated state are at least partially representative for the gene profile of microglia *in vivo*.

To compare gene profiles in cultured microglia from different donors the degree of similarity between the nine different profiles was investigated by a Pearson correlation analysis (33). As shown in Table 6.4, correlation coefficients were 0.75 on average, ranging between 0.5 and 0.9, which reflects a reasonable level of similarity. The highest correlation coefficient of 0.936 was found between the microglia gene profile of donor 8 (MS) and that of donor 6 (control). The lowest correlation of 0.521 was found between the profiles of donors 8 (MS) and 4 (AD). Together, these data indicate that gene profile as established using the current cDNA array is a stable feature in all nine microglia cultures. No obvious correlation could be discerned between the profiles and several features of the material including post-mortem delay, age or sex of the donor or clinical history of CNS disease.

Table 6.2. The twenty most abundantly expressed genes in cultured human adult microglia from different donors

	Signal intensities												
	donor 1	donor 2	donor 3	donor 4	donor 5	donor 6	donor 7	donor 8	donor 9	mean	SD[a]	CV (%)[b]	Gene code
CCL2 (MCP-1)	1267	1260	1378	1707	1415	1327	894	1621	1866	1415	287	20	M24545
thymosin beta-10 (TMSB10)	1151	747	1286	1485	1337	1447	1168	1294	1490	1267	232	18	M92381
neuromodulin (GAP43)	870	928	1011	1021	1059	1353	1147	1383	1716	1165	270	23	M25667
migration inhibitory factor-related protein 14	1041	862	1151	1013	1358	1257	750	1257	1579	1141	255	22	X06233
migration inhibitory factor-related protein 8 (MRP8)	978	780	1147	1153	1214	1031	378	1194	1508	1042	318	30	X06234
corticotrophin releasing factor receptor 1 (CRF-R)	1008	846	1140	1293	997	1015	592	973	1228	1010	208	21	X72304
endothelin-2 (ET2)	524	821	911	774	950	982	752	1081	1594	932	296	32	M65199
bone morphogenetic protein 2A (BMP2A)	940	889	1103	1161	875	899	598	958	956	931	158	17	M22489
CXCL8 (IL-8)	313	1002	745	1050	454	1402	507	1246	1640	929	457	49	Y00787
CCL3 (MIP1-α)	645	674	767	930	1053	1006	765	1180	1274	921	224	24	M23452
ribonuclease angiogenin inhibitor (RAI)	789	676	855	721	939	1071	686	1109	1211	895	198	22	M36717
interleukin-1 receptor antagonist protein (IL-1RA)	715	1243	842	720	634	1189	1121	833	636	882	240	27	M63099
bone morphogenetic protein 4 (BMP4) +BMP2B	166	711	565	795	1052	993	983	1011	1479	862	366	42	D30751 + M22490
BIGH3	991	808	845	1060	447	984	949	777	819	853	181	21	M77349
low-affinity nerve growth factor receptor (NGFR)	1257	1191	1195	1328	1265	54	1166	23	78	840	593	71	M14764
pleiotrophin (PTN) + human nerve growth factor	1067	1118	1216	1315	1196	123	1096	218	124	830	512	62	X52946 + M57399
interleukin-1 beta (IL-1β)	528	659	810	817	581	649	346	1043	991	714	224	31	K02770
Flt3 ligand (SL cytokine)	414	672	699	484	833	624	728	802	1158	713	216	30	U04806
CD147 (basigin, neurothelin)	691	797	861	701	741	621	907	593	342	695	168	24	L20471
CD40	553	700	777	553	541	751	676	840	829	691	119	17	X60592

[a]standard deviation,[b]coefficient of variation

Table 6. 3. Genes previously reported to be expressed in human microglia from control donors (without any treatment)

Name	Material	Reference	mean relative signal intensity on array (n=9)
CXCR1, CXCR2	adult	52	106
CXCR3, CXCR5	adult	53, 54	not on array
CXCR4	adult, fetal	55, 56	409
CCR2	adult, fetal	57	122
CCR3, CCR5	adult	58	not on array
CCR4, CCR6	adult	54	not on array
TNF-α	adult	59	686
TGF-β	adult	60	230
TGF-β1, -β2, -β3	adult	61	230
TGF-βR1, TGF-βR2	adult	61	158
IL-1	adult	62	446 (α, β variant)
IL-1α	adult	60	177
IL-1β	adult	60	714
IL-6	adult	60	682
IL-1R antagonist	adult	60	882
CD43	adult	63	not on array
CXCL8 (IL-8)	adult, fetal	52, 64	106
CD40	adult, fetal	65	691
CCL2 (MCP-1)	fetal	12	1415
CCL3 (MIP-1α)	fetal	66	921
CCL4 (MIP-1β)	fetal	66	374
CCR3, CCR5	fetal	67	not on array
CX3CR1	fetal	68	not on array
CSF-1	fetal	69	351
IL-1β	fetal	59	714
IL-10	fetal	70	296
IL-12	fetal	70	145 (α, β variant)
IL-15	fetal	71	118
IL-16	fetal	72	not on array
TNFαR1	fetal	73	665
IL-1RI	fetal	70	200
IL-1RII	fetal	70	513
IL-5R	fetal	70	282
IL-6R	fetal	70	220
IL-9R	fetal	70	207
CD137	fetal	74	not on array

Table 6.4. Pearson correlation analysis of gene expression patterns in cultured microglia from different donors

Clinical history[a]	Donor code		donor 1	donor 2	donor 3	donor 4	donor 5	donor 6	donor 7	donor 8	donor 9
dementia	01-002	donor 1	1.000	0.821	0.923	0.877	0.898	0.571	0.853	0.541	0.581
non-demented control	01-006	donor 2	0.821	1.000	0.888	0.891	0.808	0.594	0.847	0.544	0.574
AD	01-010	donor 3	0.923	0.888	1.000	0.885	0.888	0.596	0.833	0.570	0.616
AD	01-013	donor 4	0.877	0.891	0.885	1.000	0.892	0.545	0.826	0.521	0.602
non-alzheimer dementia	01-027	donor 5	0.898	0.808	0.888	0.892	1.000	0.618	0.878	0.606	0.700
non-demented control	01-033	donor 6	0.571	0.594	0.596	0.545	0.618	1.000	0.672	0.936	0.908
AD	01-042	donor 7	0.853	0.847	0.833	0.826	0.878	0.672	1.000	0.602	0.649
MS	03-052	donor 8	0.541	0.544	0.570	0.521	0.606	0.936	0.602	1.000	0.921
AD	03-057	donor 9	0.581	0.574	0.616	0.602	0.700	0.908	0.649	0.921	1.000

[a]AD: Alzheimer's disease; MS: multiple sclerosis

The effect of TNF-α or IFN-γ treatment on microglia gene expression profiles

Next, we investigated the response of cultured human microglia to the pro-inflammatory cytokines TNF-α and IFN-γ. Due to the limited numbers of microglia isolated from each tissue sample and the relatively large amount of RNA required for gene profiling, each culture was treated with only one type of stimulation. Five microglia cultures from different donors were treated for 48 h with TNF-α, and three cultures with IFN-γ. Microglia morphology or proliferation rates were not visibly affected over this 2-day treatment period by the addition of either cytokine.

Tables 6.5 and 6.6 present a summary of the results and highlight which genes were either induced or suppressed as compared to the untreated microglia in each of the samples. The most striking feature of the results is the lack of correspondence between responses in individual microglia cultures, contrasting the very similar profiles in each culture when left untreated. Taking into account a threshold value of 2 as a meaningful level of induction, expression of only two genes was induced in at least three out of the five cultures treated with TNF-α. These two genes were the vitamin B3 receptor (the G-protein coupled receptor HM74) and the cytokine receptor Epstein virus-induced gene 3. No other genes were found to be induced in more than two out of the five different cultures. After addition of IFN-γ, again only two genes were found to be induced in two out of the three cultures treated, viz. stem cell factor and the small inducible cytokine A1. None of the other genes expressed at higher levels in a given culture displayed a similar induction in other cultures. Some genes were even found to be induced in one culture and suppressed in another (e.g. follicle stimulating hormone receptor, IL-9 and frizzled-related FrzB) emphasizing the remarkable lack of correspondence in the changes observed.

With regard to genes whose expression was suppressed by either treatment the lack of correspondence was even more striking. No single gene product was suppressed in at least two different cultures by a factor of at least 2, despite the presence of quite a range of gene products that were suppressed in individual cultures (Table 6.6).

Table 6.5. Genes induced in different microglia cultures after treatment with TNF-α and IFN-γ

name	gene code	donor 1	donor 2	donor 6	donor 7	donor 8
Induction after TNF-α treatment						
vitamin B3 receptor (HM74)	D10923	**4.27**	1.55	1.57	**2.47**	**2.78**
interleukin-17 (IL-17)	U32659	**3.40**	1.26	0.60	1.53	1.50
erythroid differentiation protein (EDF)	J03634	**3.23**	1.34	0.94	**2.16**	1.45
CD135 (stem cell tyrosine kinase 1, STK1)	U02687	**3.20**	1.15	1.18	**2.12**	1.08
CXCL8 (IL-8)	Y00787	**3.08**	1.23	1.14	1.47	1.24
glial cell line-derived neurotropic factor (GDNF)	L19063	0.98	0.71	**13.07**	0.79	1.90
ciliary neuronotrophic factor (CNTF) isoforms B & C	A26792	0.85	0.49	**8.72**	0.75	0.81
interleukin-1 alpha (IL-1α)	X02851	0.88	1.31	**4.26**	1.38	**2.00**
colon carcinoma kinase 4 (CCK4)	U33635	1.15	0.65	**3.17**	1.15	0.59
follicle stimulating hormone receptor (FSHR)	M95489	0.69	*0.46*	**2.77**	0.87	0.88
CXCL6 (granulocyte chemotactic protein 2, GCP 2)	X78686	**2.31**	0.82	1.27	**2.82**	*0.33*
transforming growth factor-beta 3 (TGF-β3)	J03241	0.66	0.68	0.87	**2.47**	0.44
small inducible cytokine A1 (SCYA1), T-cell-secreted protein I-309	M57502	1.58	1.10	0.69	**2.41**	1.58
CXCL2 (MIP2-α)	X53799	1.80	1.64	1.16	1.97	**5.41**
interleukin-9 (IL-9)	X17543	1.15	*0.46*	1.02	1.03	**4.29**
interleukin-13 (IL-13)	L06801	*0.31*	0.91	**2.51**	0.95	**3.60**
Epstein-Barr virus-induced gene 3 (cytokine receptor EBI3)	L08187	**2.94**	1.10	11.93	**2.13**	**3.51**

name	gene code	donor 4	donor 5	donor 9
Induction after IFN-γ treatment				
stem cell factor (SCF)	M59964	**3.09**	0.60	**3.34**
small inducible cytokine A1 (SCYA1), T-cell-secreted protein I-309	M57502	**3.01**	1.14	**2.75**
B-cell growth factor 1 (BCGF1)	M15530	**2.84**	1.52	1.00
interleukin-7 receptor alpha subunit (IL-7R-α)	M29696	**2.78**	0.92	1.30
interleukin-3 (IL-3)	M14743	**2.56**	1.75	1.72
placenta growth factors 1 + 2 (PLGF1 + PLGF2)	X54936	0.91	**2.43**	1.26
transforming growth factor-beta (TGF-β)	X02812	1.51	**2.06**	1.07
prohibitin (PHB)	S85655	1.09	**2.00**	0.79
interferon regulatory factor 1 (IRF1)	X14454	1.49	0.77	**5.33**
vitamin B3 receptor (G-protein-coupled receptor HM74)	D10923	0.70	0.70	**3.00**
interleukin-1 alpha (IL-1α)	X02851	1.15	1.12	**2.49**

Values in bold indicate induction by at least a factor 2 with a signal intensity of at least 150 in treated cultures. Underscored values in italics indicate suppression by at least a factor 2 and a signal intensity of at least 150 in untreated cultures.

Table 6.6. Genes suppressed in different microglia cultures after treatment with TNF- α and IFN-γ

name	gene code	donor 1	donor 2	donor 6	donor 7	donor 8
Suppression after TNF-α treatment						
insulin-like growth factor II (IGF2)	M29645	**0.18**	0.70	10.34	2.20	0.74
interferon gamma (IFN-γ)	X01992	**0.29**	1.27	0.97	1.30	1.05
interleukin-13 (IL-13)	L06801	**0.31**	0.91	*2.51*	0.95	*3.60*
frizzled-related FrzB (FRITZ) + FrzB + frezzled (FRE)	U91903 + U24163	**0.32**	1.18	4.35	*2.28*	1.11
manic fringe	U94352	**0.33**	0.78	0.87	0.54	0.84
ephrin type-A receptor 2	M59371	0.12	**0.25**	3.49	1.98	0.83
kidney epidermal growth factor (EGF)	X04571	0.62	**0.32**	1.56	1.87	0.94
delta-like protein (DLK)	U15979	0.49	**0.33**	10.54	1.23	0.54
CD117 (proto-oncogene tyrosine-protein kinase kit)	X06182	0.61	**0.34**	**0.36**	1.40	0.72
papillary thyroid carcinoma-encoded protein	M31213	0.86	**0.35**	5.83	2.12	0.82
lunatic fringe	U94354	1.16	0.85	**0.44**	0.62	1.33
interleukin-1 receptor type I (IL-1R1, CDW121A)	M27492	0.94	0.64	**0.46**	1.61	1.17
hepatocyte growth factor activator (HGF activator)	D14012	1.52	0.71	**0.49**	1.06	0.98
growth inhibitory factor, metallothionein-III (MT-III)	D13365	1.10	0.74	**0.49**	0.58	1.34
CD114 (GCSF-R)	M59818	0.68	0.92	0.91	1.30	**0.17**
granulocyte-macrophage colony-stimulating factor receptor α	X17648	1.41	0.96	0.86	1.13	**0.26**
interferon consensus sequence-binding protein (ICSBP)	M91196	0.67	0.63	0.99	1.64	**0.29**
CXCL6 (granulocyte chemotactic protein 2, GCP 2)	X78686	*2.31*	0.82	1.27	*2.82*	**0.33**
interferon regulatory factor 1 (IRF1)	X14454	1.19	1.00	0.85	1.45	**0.48**

name	gene code	donor 4	donor 5	donor 9
Suppression after IFN-γ treatment				
thrombin receptor (TR)	M62424	**0.49**	1.16	1.50
TKT (neurotrophic tyrosine kinase receptor-related 3)	X74764	0.83	**0.38**	1.13
CXCL8 (IL-8)	Y00787	0.96	**0.39**	1.26
teratocarcinoma-derived growth factor 1 (TDGF1)	M96955	0.56	**0.40**	0.64
interleukin-17 (IL-17)	U32659	0.99	**0.42**	0.91
insulin-like growth factor binding protein 3 (IGFBP3)	M31159	0.98	**0.48**	0.89
inhibin alpha subunit (INHA)	M13981	1.18	1.59	**0.25**
smoothened, GX	U84401	0.94	1.60	**0.37**
fibroblast growth factor 8 (FGF8)	U36223	1.41	0.66	**0.37**
Flt3 ligand (SL cytokine)	U04806	0.72	0.69	**0.43**
manic fringe	U94352	0.55	1.21	**0.43**

Values in bold indicate suppression by at least a factor 2 and a signal intensity of at least 150 in untreated cultures. Underscored values in italics indicate induction by at least a factor 2 and a signal intensity of at least 150 in treated cultures.

Discussion

Much remains to be established about the way microglia respond to and participate in the communication network of cytokines, chemokines and growth factors in the human CNS. Examining cultured microglia from human post-mortem brain appears an attractive approach to clarify their cell biological functions. So far, however, it has remained unclear to what extent such cultured microglia behave in a reproducible manner or to what extent they are representative for microglia in an intact CNS *in vivo*. In this study, we attempted to address these issues by a comprehensive analysis of the profile of cytokine, chemokine and growth factor gene expression in cultured microglia derived from different donors both before and after treatment with pro-inflammatory cytokines.

Both in their morphology and in their gene expression cultured microglia reflect a distinct state of activation as compared to resting microglia *in vivo*. Cells are bi-polar and express high levels of the activation-associated chemokines CCL2, CCL3 and CXCL8 along with the activation markers MRP8 and MRP14, that are undetectable in resting microglia *in vivo* (34,35). Clearly, cultured microglia are different from the highly ramified resting microglia in normal brains and spinal cord. This is hardly surprising since microglia are highly responsive and will inevitably undergo some form of activation as the result of mechanical and metabolic stress during isolation, confrontation with serum components and growth factors in the culture medium, and of elimination of CNS-borne silencing signals (36). The effects for example of GM-CSF (present in our culture medium) on the gene profile of macrophages (37) and neonatal murine microglia have previously been documented (38).

Under our standard culture conditions, gene expression profiles of microglia from different donors are very similar. This common profile reveals two important features of cultured microglia. First, several of the mediators expressed at relatively high levels appear to be mediators of neuroprotection and regeneration. Expression of neuromodulin, endothelin-2, bone morphogenetic proteins 2A, 2B and 4 and pleiotrophin along with the low-affinity nerve growth factor receptor are clear clues that microglia may very well actively participate in signaling functions involved in controlling repair rather than only producing a polarized pro-inflammatory reaction. This is fully in line with the increased awareness that microglia are not just mediators of host defense and antigen-presentation, but exert direct protective functions in the CNS as well.

Secondly, the mediators found at the highest levels fully correspond to gene products previously documented by others in human microglia either *in vivo*

or *in vitro*. Table 6.3 lists cytokines, chemokines and growth factors previously reported by others to be expressed in partially activated human microglia. To the extent that these products were represented on the array used in this study, all products could be readily detected and were found to be expressed at levels of at least 10 % of that of the mean of the house-keeping genes used as reference. This not only confirms the validity of the cDNA read-out assay as such, but also indicates that cultured microglia at least partially reflect microglia as found *in vivo*.

In sharp contrast to the reproducible gene expression patterns of microglia from different donors when cultured under standard conditions, treatment with the pro-inflammatory cytokines TNF-α or IFN-γ led to a widely diverse response in each individual culture. None of the 268 genes displayed a consistent induction or suppression in all cultures tested and often, even opposing effects were observed in that certain gene expression signals increased in one culture while decreasing in another under the same experimental condition. Technical issues are highly unlikely to have caused this variation since reproducibility of the cDNA array analysis in its own right is confirmed not only in the first part of the present study, but has been firmly established also in a previous study of gene expression profiles in astrocytes using the same technology (18).

Only limited data on the response of human microglia to TNF-α or IFN-γ are available to compare our results to. One of the best documented responses to these mediators includes elevated production of IP-10 (39) but unfortunately, IP-10 is not represented on the array used here. Hua and Lee also reported induction of CXCL8 (IL-8) by TNF-α but not by IFN-γ. This appears to be largely in line with our data showing an overall lack of induction of CXCL8 by IFN-γ, and marked induction of CXCL8 by TNF-α in one donor (3-fold induction) and some induction (up to 1.5-fold) in the others. Interestingly, also Hua and Lee already noted marked variation in their data between microglia cultures from different donors, yet did not elaborate on this issue. A recent cDNA-based analysis of the murine microglia response to the combination of TNF-α and IFN-γ has revealed a large variety of genes being induced by the stimulus including several genes that are also represented on our array (40). The correspondence between these data on murine microglia and ours on human microglia is generally very poor, in this case possibly also due to species differences. A comparison between the response to TNF-α/IFN-γ for murine and human microglia, and especially a comparison between the different cultures analyzed in this study thus reveal a striking lack of consistency. We interpret this to signify real biological variations among the different

microglia cultures that only manifest themselves upon a challenge with pro-inflammatory signals, and that remain latent as long as the microglia are kept under standard culture conditions. A very similar set of observations have recently been reported for gene profiling studies of activated murine microglia (41,42). Again, strikingly heterogeneous gene expression patterns were monitored for different activated microglia despite a consistent methodological approach. In a study of the effects of the beta amyloid peptide 1-42, Walker and co-workers also performed gene profiling analysis but in their approach RNA samples from five different microglial cultures were pooled prior to the analysis (43), unfortunately leaving open the issue of donor-to-donor variations.

In our study, no obvious link could be discerned between the data and several features of the microglia source such as post-mortem delay, age, sex or clinical history of the donor. Different anatomical origins of the microglia samples are, however, a possible source of the variation. Production of neurotrophins and inflammatory mediators including IL-1, IL-6, nitric oxide and metallothionein, expression of MHC molecules as well as the response to brain insults *in vivo* differ from one region of the brain to another (42,44,45,46,47,48). Along with the fact that microglia densities across the CNS can vary considerably (49,50) this contributes to differential responsiveness and functionality of different brain regions in infection and trauma. The precise anatomical origin of the microglia cultures examined in the present study has not been documented in detail thus precluding any further analysis of this parameter. Another factor that affects microglia functions is age (48,50,51). Yet, the influence of this factor is prominent at early stages of mammalian life but much less documented for later stages. The way age differences in our donor group between 50 and 90 could have affected microglia performance remains to be clarified. Finally, microglia from different regions of the brain could proliferate at different rates under the culture conditions applied here. This could well lead to a certain level of skewing in the final population of cells that are subjected to gene profiling after being kept in culture for 1-2 weeks in order to obtain the required level of phenotypic purity.

When taken together our data indicate that cultured human microglia from different donors represent populations of partially activated cells with a stable gene expression profile, irrespective of the post-mortem delay, anatomical origin or clinical features of the donor. Yet, when further treated with TNF-α or IFN-γ, microglia from different donors respond very differently. This variation does not appear to be a methodological artifact but a biological reality that requires further examination.

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CHAPTER 7

General Discussion

The main goal of the studies described in this thesis was to investigate the immune modulatory effects of human herpesvirus-6 (HHV-6) infection on cultured human adult glial cells. For many years virus infections have been thought to be associated with the development of MS. Among a wide range of viruses (as discussed in Chapter 1) HHV-6 and Epstein-Barr virus (EBV) have been particularly suspect of an involvement in MS. For HHV-6, elevated levels of viral protein have been demonstrated in CNS tissue, lymphoid tissues, peripheral blood lymphocytes, serum, saliva and urine from MS patients as compared to healthy controls (1,2,3,4,5,6,7,8,9). Yet, not all studies have confirmed such a difference between MS patients and healthy controls (10,11,12,13,14).

To understand the possible impact of HHV-6 on CNS inflammation such as occurs in MS, it is necessary to clarify the cell-biological effects of viral infection on glial cells. These effects may very well involve modulation of inflammatory reactions since the HHV-6 genome encodes multiple gene products that interfere with chemokine signaling in infected target cells. The U12 region of the viral genome encodes a beta chemokine receptor for CCL2, CCL3, CCL4 and CCL5, the U51 region encodes a CCL5 receptor and the U83 region encodes a chemokine-like CCR2 agonist (15,16,17,18). HHV-6 infection might therefore well alter host cell responses to inflammation in ways that are dependent on the type of host cell and its state of activation. A few examples have already been identified in which HHV-6 infection changes the behavior of the target cell. Infection of human macrophages with HHV-6 impairs the ability of macrophages to produce IL-12 in response to IFN-γ (19), most likely by inducing high levels of IL-10 in the infected macrophages (20). In a T-cell line designated SupT1, HHV-6 infection induces expression of several pro-inflammatory genes but in this case, suppression of anti-inflammatory gene products including IL-10 was noted (21). Thus, while HHV-6 does appear to interfere with cytokine signaling by the target cell, the way this works out may very well be different from one type of cell to another. Essentially nothing is known on the effects of HHV-6 on gene expression in glial cells that are key in controlling inflammatory reactions in the CNS.

While the association of HHV-6 with MS is still controversial EBV is clearly associated with MS. This association is based on the observations that essentially all MS patients (99 to 100 %) are EBV seropositive versus 90 to 95 % of the general population, and that anti-EBV immunoglobulin (Ig) serum titers are higher in MS patients (22,23,24). Furthermore, a delayed primary infection with EBV resulting in a history of infectious

mononucleosis is associated with a significantly increased risk of MS later on in life (25,23). Intriguing is the fact that EBV infection in B cells enhances the expression of αB-crystallin, a small stress protein that has previously been identified as an immunodominant antigen in CNS myelin of MS patients (26). The impact of EBV infection on glial cells is similarly still unclear. In our own experiments, human adult astrocytes in culture could be infected with EBV but levels of infection (i.e. the percentage of infected astrocytes) were too low to allow for a reliable analysis of the effects of EBV on the gene profile.

In present study we focus on the effects of HHV-6 on glial cells. To clarify the role of HHV-6 in neurodegenerative diseases such as MS we examined its impact on immune regulation of cultured human adult astrocytes. Both normal cultured astrocytes were used and astrocytes provoked by a mixture of the cytokines TNF-α, IL-1β and IFN-γ that mimics an inflammatory microenvironment such as exists in active MS lesions (27). It is well known that increased levels of TNF-α, IL-1β and IFN-γ are present in active MS lesions, and in MS patients levels of these cytokines in CSF and serum correlate with disease progression (28,29,30,31). TNF-α is present in acute and chronic active MS lesions but absent from chronic silent lesions (32). Increased levels of TNF-α produced by $CD4^+$ T-cells correlate with long-term increase in lesion load in MS patients as assessed by MRI (33). More recently, detailed gene expression analysis have confirmed elevated levels of IFN-γ in active MS lesions (34). Thus, examining astrocytes in the presence of all three pro-inflammatory cytokines will help reveal how the cells might behave under conditions of active inflammation in an intact brain.

In vitro studies of human adult glial cells

The current availability of well characterized post-mortem human brain tissue provides the opportunity to culture different human adult glial cell types such as astrocytes, microglia and oligodendrocytes. The use of primary human adult glial cells offers an advantage over the use of transformed immortalized cell lines since primary cells are expected to represent more closely the behavior of glial cells *in vivo*. Obviously, cell cultures are different from an intact human brain in lacking a variety of intercellular and matrix-derived interactions. Nevertheless, they do provide unique possibilities to approach different experimental conditions that are impossible to examine in humans notably including experimental virus infection.

Throughout our studies, we have used cultured human adult post-mortem astrocytes and microglia obtained from white matter brain samples as described previously (Chapter 2 and 6). Post-confluent monolayers of cultured human adult astrocytes were used at passage four. Astrocytes cultured up to eight passages show a star-shape morphology and proliferate well. After passage eight cells become sensitive to the CD95/CD95L apoptosis pathway and tend to enter apoptosis (35). Human microglia were grown in medium supplemented with granulocyte macrophage colony-stimulating factor (GM-CSF) and used within 2 weeks after isolation at a time when essentially pure cultures are obtained. The addition of GM-CSF as a growth factor may seem artificial but it should be kept in mind that GM-CSF is expressed by both human adult astrocytes and microglia. GM-CSF is also expressed by astrocytes in MS lesions and the growth factor therefore represents a biologically relevant stimulus (36). Cultured microglia have a bi-polar morphology and represent partly activated cells similar to what can be expected during inflammatory activation in the CNS such as occurs in MS.

There are several practical differences between astrocyte and microglia cultures that need to be accommodated. The yield of astrocytes, using a given quantity of brain tissue, is higher as compared to the yield of microglia. Astrocytes proliferate faster and can easily be frozen and thawed which makes it possible to culture large numbers of cells. Therefore, it is also possible to investigate different experimental conditions using separate astrocyte cultures derived from a single donor. Microglia on the other hand are more difficult to obtain, they cannot be frozen and the possibilities to investigate different stimuli on microglia derived from a single donor are limited.

Communication between glial cells and their environment occurs besides physical interactions via a wide range of mediators such as cytokines, chemokines, growth factors and their receptors. Rather than focusing on a few selected cytokine reporter genes in an attempt to understand the impact of viral infection on glial gene profiles, we used a cDNA gene profiling array containing 268 gene sequences encoding cytokines, chemokines, growth factors and their receptors. Each of these 268 genes represents a part of the intricate network of cytokines and chemokines, each of which could possibly be influenced by the virus. In this way, a broad view could be obtained on the impact of viral infection on glial cell functions without having to resolve to high-density arrays which would have posed a serious data processing challenge as well as a financial challenge. To allow ourselves the more or less routine use of this array as a gene profiling read-out instrument, we first investigated the reproducibility of the data

generated from repeated astrocyte cultures obtained from a single donor, and also from astrocyte cultures from different donors (as described in Chapter 2). Apart from the technical performance of the gene array itself, major issues to be addressed included whether the gene profiles obtained were not only reproducible as such, but also representative for glial responses *in vivo*. Below these issues are discussed to illustrate that in our view gene profiling of cultured human glial cells as performed here does indeed provide a reliable and representative image of the behavior of the cells in the human brain.

Gene profiles of cultured astrocytes and microglia are highly reproducible

To address the issue of reproducibility we first examined gene profiles of separately cultured astrocytes samples that had all been derived from a single donor. In total, eight of such samples were compared. Furthermore, gene profiles were recorded of a further two astrocyte cultures, each from two different healthy donors. As described in Chapter 2 the gene expression profiles obtained in this exercise show a remarkable agreement both amongst the eight separate astrocyte cultures from the same donor as well as amongst astrocytes from different donors. This confirms not only that the gene profiling technique as such is reproducible, but also indicates that gene expression profiles are very similar between astrocyte cultures from different donors.

In Figure 7.1 the coefficient of variation (CV; standard deviation expressed in percentage) is plotted against the strength of the gene expression signal for each of the 268 genes analyzed in the eight astrocyte cultures from a single donor. As can be expected, higher gene expression signals are characterized by relatively low CV values and *vice versa*. It is unlikely that the CV values are significantly influenced by variations in the hybridization step of the analysis (when radioactive cDNAs are blotted onto the filter) since the duplicate gene hybridization signals that we have monitored throughout our studies are always extremely reproducible. Most likely, high CVs for low signals merely reflect a general relationship between the reliability of quantification and the signal-to-noise ratio also since a similar curve is obtained for the relationship between CVs and gene expression signals in nine different microglia cultures (Figure 7.3). Figure 7.2 shows the relationship between the coefficient of variation as observed for gene expression signals in astrocytes derived from different donors. Interestingly, CV values associated with this comparison tend to be somewhat lower than those represented in Figure 7.1. Clearly, it is highly unlikely that gene profiles of astrocytes obtained from different donors would resemble each

other more than gene profiles of separate astrocyte cultures from a single donor. More likely, the difference can be accounted for by the fact that the gene profiles on which Figure 7.2 is based were analyzed on the same day while the data on which Figure 7.1 is based were collected on different days. Thus, slight technical variations will affect the result in a noticeable manner, albeit that such variations are not expected to selectively affect a few genes only. Nevertheless, critical comparisons of gene profiles are therefore best performed using gene profiles analyzed in parallel at the same time. In particular in Figure 7.1, reflecting a comparison of eight astrocyte gene profiles obtained from a single donor, a few genes deviate markedly from the general relationship between CV and gene expression signal strength. These few genes, whose detection is accompanied by relatively large coefficients of variation, include IL-4, IL-6, BMP2A and endothelin-2. Interestingly, these genes are highly inducible in astrocytes. BMP2A is induced upon TNF-α treatment, as described in Chapter 2, while IL-6 is induced upon cytokine treatment of HHV-6-infected astrocytes, as described in Chapter 4. Also data from others - as reviewed in Chapter 2 - confirm that IL-4, IL-6 and BMP-2A are easily inducible in human astrocytes (37,38). Therefore, it is likely that these particularly inducible genes easily respond to subtle variations in cell culturing conditions or cell density which results in the unusually high coefficients of variation. Such an apparent intrinsic variability for certain genes in certain types of cells is useful to keep in mind when comparing the effects of certain stimuli on gene profiles.

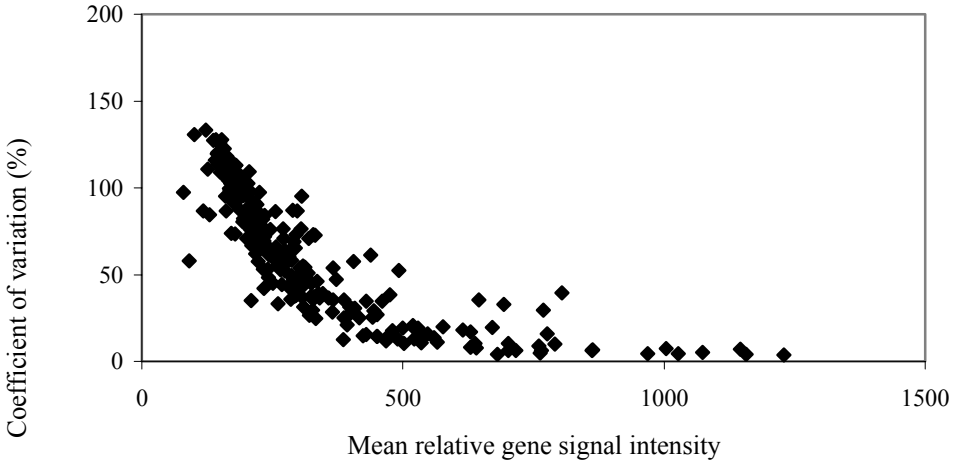

Figure 7.1. mean relative signal intensities and their coefficients of variation of 268 genes from eight array experiments using astrocytes from one single donor

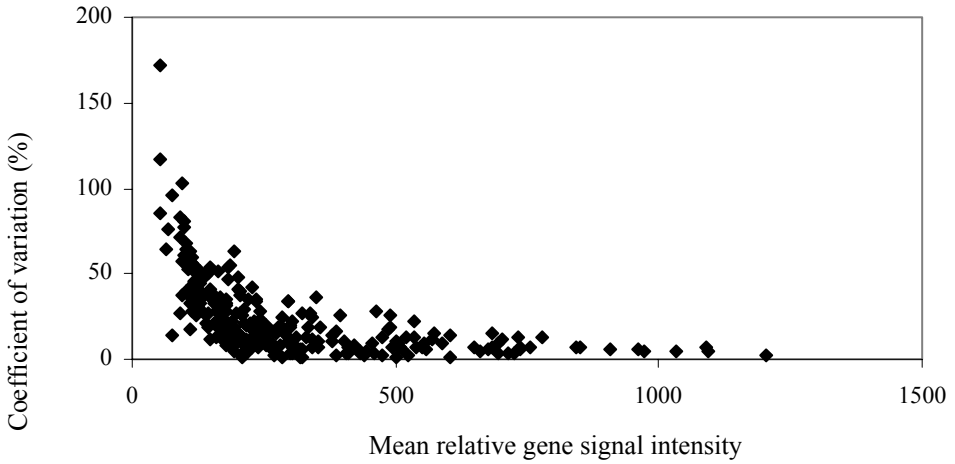

Figure 7.2. mean relative signal intensities and their coefficients of variation of 268 genes expressed in astrocytes from three different donors

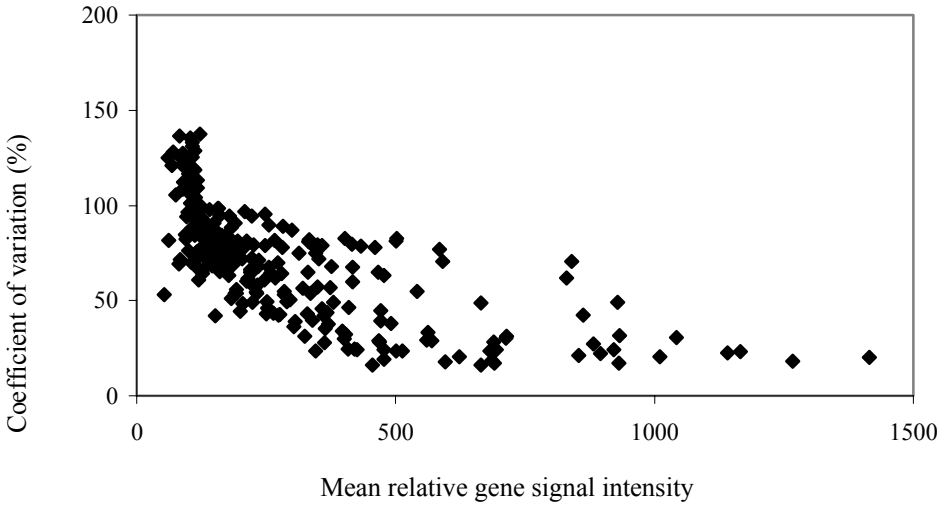

Figure 7.3. mean relative signal intensities and their coefficients of variation of 268 genes expressed in microglia from nine different donors

Another important issue to consider is the question to what extent the changes we have observed in gene profiles of cultured glial under various conditions cells mirror the behavior of astrocytes and microglia *in vivo*. First of all, the changes in the expression of cytokines and chemokines we have monitored are very similar to what others have observed in cultured human glial cells, while using different samples and different analytical techniques. Almost without exception, genes that have been reported by others to be induced in cultured glial cells and that are included on the array, are also found to be induced by our approach. This comparison is elaborated on in detail in Chapters 2 and 6. Clearly, validating the results of any gene profiling study could be performed by verifying certain gene induction data using RT-PCR, or by establishing increased protein levels by ELISA or immunocytochemistry. Yet, in an approach such as ours the question arises for how many genes the expression data should be verified for any validation to be sufficiently thorough. In view of the above excellent correspondence between our data and those reported by others, we made no attempts to validate individual gene expression signals by alternative technologies. We feel strengthened in this attitude by comparing our data to all the available data that have been published on cytokine and chemokine

expression by astrocytes and microglia in (inflamed) human brain tissue. A variety of studies, using different brain samples and different analytical techniques and reagents provides a relatively broad framework of data to compare our results to. As elaborated in detail in Chapters 2 and 6, this comparison shows that essentially all cytokines or chemokines that have been documented by others to be expressed by glial cells in inflamed human brains, and that are represented on the cDNA array, are also found in cultured and partially activated glial cells. This consideration goes beyond the question of technical validation and appears supportive for the idea that cultured glial cells indeed mirror the behavior of glial cells in an intact (inflamed) human brain to a sufficient extent to be considered as a useful model.

As an extension to the above statement, it should be noted that the data in Chapter 6 do reveal an unexpected and interesting aspect of microglia cultures. Despite the fact that microglia cultures from different donors appear to have similar gene profiles under standard culturing conditions, their response to a pro-inflammatory signal varies widely (see also below). It is unknown as yet to what extent this may reflect actual differences between microglia residing in different parts of the brain, or being derived from different donors. We do not consider it to be an experimental artifact and take this observation as an encouragement to more closely examine the possibility of regional differences in microglial responses also in an intact brain.

When taken together, our data appear supportive for the idea that cultured human astrocytes and microglia largely behave reproducibly, except for the unusual diversity in microglia responses to pro-inflammatory signals. The use of a low-density cDNA array as described by us produces data that are very much in line with data obtained by others and, more importantly, they are very much in line with data on astrocyte and microglia responses in an intact human brain. Using cell culture models, therefore, relevant data may be obtained that will help understand the way glial cells behave in an intact brain under a variety of conditions.

Detection of astrocyte signaling genes as well as novel gene products

The twenty most abundantly expressed genes in astrocytes (described in Chapter 2) include genes that are mediators primarily involved in proliferation, growth and differentiation as well as their receptors. These are the low affinity nerve growth factor receptor (NGFR), insulin-like growth factor binding protein 2 (IGFBP2), thymosin beta-10 (TMSB10), pleiotrophin (PTN, involved in growth and migration), connective tissue growth factor (CTGF), vascular endothelial growth factor (VEGF) and its

receptor, insulin receptor (INSR) and epidermal growth factor receptor (EGFR). Furthermore, TNF-α and IL-6 are abundantly expressed consistent with their role in growth and neuronal survival, apart from their involvement in inflammatory reactions (39). The chemokine CCL2 is also abundantly expressed in astrocytes, along with its receptor. This expression is consistent with a key role of CCL2 as an autocrine migratory factor for astrocytes during gliosis (40). As also explained below, this emphasizes that post-confluent astrocyte cultures such as the ones used in our studies can be considered to mimic a state of gliosis *in vitro*.

The data collected by us reveal expression of several genes that to the best of our knowledge have not previously been documented for astrocytes. These novel astrocyte gene products include IL-17, CD70 (CD27 ligand), CD147 (basigin, neurothelin), BIGH3, UFO/Axl receptor tyrosine kinase and TEK/TIE-2 receptor tyrosine kinase. Especially the astroglial expression of IL-17 is an interesting observation since this cytokine has hitherto been regarded as a powerful pro-inflammatory mediator that is specifically expressed by memory T-cells (41). That IL-17 in MS lesions would be produced by such memory T-cells is already questionable since IL-17 is expressed at much higher levels in chronic inactive MS plaques as compared to early active lesions, inconsistent with T-cells being the prime source (34). Our data suggest that astrocytes could in fact be responsible for marked IL-17 production at these late stages of MS plaques. The presence of IL-17 in inactive plaques (but not active plaques) along with the notion that at this stage astrocytes are primarily involved in controlling inflammation appears at odds with a strictly pro-inflammatory character of IL-17. More likely, astrocyte-derived IL-17 may play a role in down-regulating inflammation and/or promoting repair. Together, our data therefore suggest that IL-17 may not only be produced by cells other than just memory T-cells, but also that it may have dual functions dependent on its context. The recent interest in IL-17 as a target for therapy in MS maytherefore require revision.

Impact of pro-inflammatory cytokines on astrocyte gene profiles: Limited synergistic effects

In Chapter 2, we described the effects of an inflammatory mimic consisting of TNF-α, IL-1β or IFN-γ on the astrocyte gene profile. Cytokine treatment of post-confluent astrocyte monolayers had no effect on the morphology of the astrocytes. In general, TNF-α and -to a lesser extent- IL-1β lead to induction of several genes whereas IFN-γ has very little impact on expression levels of the set of 268 genes represented on the array used. As a rule, any effect of these cytokines involved gene induction and no genes were found to become down-regulated by any of the cytokines.

TNF-α treatment of astrocytes resulted in increased expression of several chemokines including CCL2 (MCP-1), CCL4 (MIP-1β), CCL5 (RANTES), CXCL6 (GCP 2) CXCL8 (IL-8) and CXCL9 (MIG). Furthermore, growth and neuroprotective factors were induced such as BMP-2A, BMP-3, neuromodulin (GAP43), BDNF and G-CSF and receptors including the CRF-receptor, the calcitonin receptor (CTR) and TKT. The response to IL-1β involves largely the same range of genes, but responses were blunted in comparison to the response upon TNF-α treatment. One of the more prominent responses of astrocytes to either cytokine is production of strongly increased quantities of CXCL8. As a matter of exception, we verified increased CXCL8 expression under such conditions by confirming elevated levels of CXCL8 in the culture medium using ELISA (data not shown). CXCL8 is an interesting response marker of astrocytes that is activated by a variety of stimuli and appears to contribute to the resistance of astrocytes to CD95/CD95L (Fas/Fas ligand)-mediated apoptosis. Upon triggering CD95 on astrocytes CXCL8 and its receptor CXCR2 are both induced and they are associated with an increased resistance to apoptosis, apparently as part of a self-defense response by astrocytes (42).

Astrocytes treated with a mixture of all three cytokines together displayed responses that are largely similar to those found in response to TNF-α or IL-1β alone and little indication was obtained for any synergistic effects, despite reports by others that appear to indicate otherwise (43,44,45,46). We observed only a few apparently synergistic effects in that IGFBP3, CXCL2 (MIP2-α), ephrin A3, follistatin-related protein, neurotrophin-3, IL-11, IGF-2 and erythropoietin receptor were found to be induced by the mixture of cytokines while being less affected by each of the cytokines individually. Most of these effects, however, were rather modest. The cytokine IL-11 for example, which is discussed below in more detail since it is strongly induced upon cytokine treatment of HHV-6-infected astrocytes, was

induced by a factor of only 3 by the cytokine mixture, and by a factor of 1.4 by IL-1β alone. Overall, responses to the mixture of cytokines were quantitatively less as compared to responses to either TNF-α or IL-1β alone, for reasons that are not fully understood.
Lack of any striking synergy between the three pro-inflammatory cytokines appears consistent with a number of astrocyte features observed in our studies. First of all, treatment of astrocytes with the individual cytokines as well as with the cytokine mixture had no influence on expression levels of the receptors for TNF-α, IL-1β or IFN-γ, indicating that their sensitivity towards each cytokine remains similar. Secondly, astrocytes respond rather slowly to any stimulus, rendering it unlikely that any indirect effect mediated by a responsive gene can exert its influence within the 48 h-time frame of most experiments. As detailed in Chapter 2, changes in astrocyte mRNA levels upon cytokine treatment develop and accumulate over periods of days rather than hours. This repeated observation is fully in line with previous reports by others in which cytokine-induced chemokine responses in human fetal astrocytes at the level of mRNA consistently took 48 to 72-h periods to fully develop (46). Therefore, while synergistic effects between cytokine-induced changes may well occur at later stages, they do not appear to influence astrocyte responsiveness in the first 2 days.

HHV-6 infection impacts marginally on the astrocyte gene profile
So far only four studies have been published documenting experimental HHV-6 infection in cultured human glial cells including primary astrocytes, oligodendrocytes and microglia (47,48,49,50). In these studies glial cells were infected using HHV-6-infected T-cell lines as well as cell-free HHV-6 (free from any viable cells or sub cellular particles). He et al. reported that infecting human fetal astrocytes using a HHV-6-infected T-cell carrier line in stead of cell-free virus resulted in more rapid formation of syncytia (47).
In these previously reported studies on HHV-6-infected glial cells the percentages of infected cells have remained undocumented. Yet, to reliably study the impact of infection on astrocyte gene profiling it is important to have a substantial part of the cultured astrocytes stably infected with HHV-6. In our studies, we succeeded to achieve reproducible and stable infection of up to 35 % of all cells in a given astrocyte culture as determined by staining for HHV-6 protein, as described in Chapter 3. Improving on this level of infection by co-culturing larger numbers of infected carrier T-cells, or by culturing the infected astrocytes for periods longer than 3 days proved impossible. Culturing the HHV-6-infected astrocytes for longer periods for example resulted in rapid expansion of uninfected astrocytes since these

proliferate faster than infected ones. Since the effectiveness of infection was quite different between the two T-cell lines used as a source of virus, increased levels may perhaps be obtained using yet other T-cell lines. Alternatively, it is conceivable that HHV-6-infected astrocytes may be separated from uninfected ones by selecting for increased cell size that typifies syncytia, or by employing certain flowcytometric markers.

When examining the effects of HHV-6 on astrocytes, the first striking observation in our studies was that HHV-6 infection impacted only marginally on the cytokine and chemokine profile of the astrocytes. After HHV-6 infection of cultured astrocytes only leukocyte interferon-inducible peptide, CCL5, IGFBP6 and VEGF-C were markedly induced. Furthermore, a few genes were suppressed including trk-T3, IGFBP complex acid labile chain, ephrin type-A receptor 5, ephrin-B2, TGF-β3, colon carcinoma kinase 4 and transmembrane receptor PTK7. In our view, it is unlikely that these findings would have been much different if the analysis would have been performed at times different from our more less standard 48-h analysis time. Also in monocytes for example, cytokine responses to HHV-6 infection (if any) required 2 to 5 days to develop (51). Furthermore, as stated above already, astrocyte responses to any stimulus develop at the level of mRNA at a time scale of days rather than hours. One could argue that the lack of any major response to HHV-6 infection largely reflects the fact that only 30-35 % of the cells are infected and that any potential response is diluted by gene expression signals of uninfected astrocytes. Yet, when this is corrected for by using a threshold of an induction factor of 1.6 rather than 2.0 to evaluate changes, still no marked changes are observed. Also, the response of infected astrocyte cultures to cytokines, as described below, demonstrate that even when representing a minority of the cells, HHV-6 infected astrocytes can very well impact in quite a dominant way on (changes in) the overall gene profile.

It is of interest to compare our data on the gene profile in HHV-6-infected astrocytes to changes in HHV-6-infected T-cell lines. This comparison that is presented in Chapter 4 reveals striking cell-specific features, also by revealing that none of the changes in gene expression induced by HHV-6 in the SupT1 T-cell line (21) could be reproduced in the HSB-2 T-cell line used by us. Studies by others indicate that expression of CCL2, CCL3, CXCL8, IL-10, IL-12 and IFN-γ is enhanced in HHV-6-infected human endothelial cells, hepatoma cell line or monocytes as assessed by PCR and ELISA (20,51,52,53,54). Yet, none of these genes were induced in infected astrocytes. Clearly, HHV-6 has widely different effects in different types of target cells.

Inflammatory conditions change the silent HHV-6 infection

In strong contrast to the limited impact of HHV-6 infection on the astrocyte gene profile is the dramatic impact of infection on the response by astrocytes to pro-inflammatory cytokines. While hardly or not at all induced by pro-inflammatory cytokines in normal astrocytes, infected astrocytes displayed strongly elevated levels of IL-10 and IL-11, both powerful anti-inflammatory cytokines. Apart from its well-known immune-regulatory effects on T-cells IL-10 also regulates the cytokine network in microglia by inhibiting expression of the pro-inflammatory cytokines IL-1β and TNF-α as well as of the receptors for IL-2 and IL-6 (55). Both IL-10 and the similarly anti-inflammatory IL-11 are known to down-regulate expression of the co-stimulatory molecules CD80, CD86 and CD40 as well as MHC class II on microglia, thus frustrating their ability to activate infiltrated T-cells (56,57,58,59). Also, a remarkable collection of chemokines are induced in HHV-6 infected astrocytes by TNF-α, IL-1β and IFN-γ, different from uninfected cells. These genes including CCL3, CCL5, CXCL2 and CXCL6 are important mediators in the communication between astrocytes and microglia and play key roles in their migration. Cytokine treatment of HHV-6-infected astrocytes also resulted in induction of type I interferon response regulating genes such as leukocyte interferon-inducible peptide, interferon regulatory factor 1 and interferon consensus sequence binding protein. Induction of these genes is consistent with the notion that a type I interferon response is generally geared towards combating viral infection, though not necessarily involving an adaptive immune response. Furthermore, many growth-related genes were induced such as neuregulin, VEGF, metallothionein along with the growth- and immune-modulatory genes IL-1β and IL-6. As elaborated in Chapter 4, the set of growth-related genes that are induced in infected astrocytes was completely different from the one induced in uninfected astrocytes.

Still much is to be learned on how certain cytokines, chemokines and growth factors influence glial cells functioning, and how they could influence each other in this respect. Therefore, it is near to impossible to judge the overall significance of the changes effected by HHV-6 at face value. Yet, we consider it to be quite remarkable that at least two of the best known anti-inflammatory cytokines, viz. IL-10 and IL-11, with proven immune down-regulatory qualities are among the products triggered in HHV-6-infected cytokines by a pro-inflammatory stimulus. We therefore interpret this as an indication that HHV-6 infection of astrocytes is more likely to result in local immune-regulatory reactions rather than pro-

inflammatory reactions. In other words: based on these data HHV-6 infection of astrocytes is not very likely to activate a local inflammatory response but rather tends to down-regulate any response that might emerge as the result of other factors. Or in yet other words: in astrocytes HHV-6 promotes an immunologically suppressive environment.
It is remarkable that HHV-6 infection accounts for such a dramatic change in gene profiles after cytokine treatment despite the fact that only about 30 % of the astrocytes are infected. It is unlikely that gene expression data are influenced by viral spread upon cytokine treatment or other indirect effects. First of all, no signs of virus release from infected astrocytes are visible in culture and it is known that in oligodendrocyte cultures such also does not occur (48). Infected astrocytes engage in syncytium formation and can all be stained for HHV-6 protein. No individual astrocytes outside syncytia were ever found to contain HHV-6 protein and cytokine treatment had no visible impact on these features over the 48-h monitoring period. Secondly, it is unlikely that any newly released virus would effectively infect novel target cells since our experience with cell-free HHV-6 preparations as described in Chapter 3 indicates such cell-free virus to be highly ineffective in this respect. Thirdly, indirect effects on gene expression caused by mediators from the infected astrocytes are also unlikely to occur, given the timeframe of astrocyte responses. Given these considerations, we take the data on gene expression profiles in HHV-6-infected cultures to indeed reflect changes in the 30-35 % infected cells only. The impact of cytokine treatment on these gene profiles is therefore much stronger as compared to uninfected astrocytes and essentially overrules them, in all three experiments performed. This was also found with infected astrocytes treated with the individual cytokines separately as described in Chapter 4 which produced consistent data and again pointed to very limited synergistic effects between these cytokines.

HHV-6 infection and cytokine treatment impact on TLR expression in astrocytes

To explore the role of TLR in the host response to neurotropic infections we investigated the impact of HHV-6 infection as well as the pro-inflammatory cytokines TNF-α, IL-1β and IFN-γ on expression in astrocytes of the TLR family members 1 through 4. These family members were monitored since previous data thought us that TLR5 to 10 are hardly expressed at all in astrocytes. Also, the combination of HHV-6 infection and cytokine treatment was examined for its effects since in Chapter 4 it is illustrated that HHV-6 exerts very different effects on astrocytes under normal *versus* pro-inflammatory culturing conditions. In agreement with our previous studies

(60) we found that also in the cultures studied in the present context astrocytes express high levels of TLR4 while TLR3 was less abundantly expressed, followed by TLR1 and TLR2. HHV-6 infection of astrocytes led to a preferential and strong increase (10- to 200-fold) in expression levels of TLR3-encoding mRNA while the expression levels of TLR1, TLR2 and TLR4 were only modestly increased (less than 10-fold). Infection of the astrocytes with HHV-6 did not result in any suppression of TLR expression. Treatment of astrocytes with either IL-1β, TNF-α or IFN-γ, as well as with a mixture of these cytokines resulted in changes in TLR expression levels similar to those triggered by HHV-6 infection and again, in preferential induction of TLR3. Interestingly, the combined effects of cytokine stimulation and HHV-6 infection appeared to induce higher levels not only of TLR3 again (5- to 100-fold), but in this case induction was observed also of TLR2, between 10- to 100-fold. Changes in TLR1 or TLR4 expression were much less marked. The data, however, also reveal significant donor to donor variations that render it difficult to draw any far-reaching conclusions based on the more or less preliminary set of data generated in the current study. Extension of our data by analyses of more astrocyte samples will be required to confirm preferential induction of TLR3 by both HHV-6 and cytokines, and the additional induction of TLR2 by cytokines in infected astrocytes.

Intuitively, one might expect viral infection to trigger enhanced expression of TLR that are specifically suited to recognize viral structures and help mount a host defense response against it. In this case, however, such does not seem to be the case. Generally, engagement of TLR including TLR3 and TLR2 activates intracellular signaling pathways that trigger enhanced production of IL-1β, TNF-α and IL-6, among a variety of other responses. While different TLR do activate different sets of responses, these mediators are invariably part of these responses, notably also in astrocytes (Bsibsi et al, unpublished data). As described in Chapter 4 however, HHV-6 infection of astrocytes does not result in any significant induction of either one of these common TLR-responsive gene products, whereas they would be expected to become detectable at least to some extent in the 48-h time frame of the experiment in case they were triggered. This strongly suggests that while HHV-6 does induce elevated levels of TLR3, the virus does not trigger its activation within the time frame of the experiment. This appears to be in line with the current knowledge on TLR3 agonists. So far, only double-stranded RNAs (and certain double-stranded segments of mRNAs or siRNAs) are known to activate TLR3 and it is not obvious how HHV-6 infection could provide these ligands to surface-exposed TLR3 receptors on

astrocytes. Intriguingly, therefore, HHV-6 infection of astrocytes appears to prime the infected cells to become responsive to TLR3 ligands that are probably not derived from HHV-6 itself.

Differences in microglia gene profiles upon inflammatory cytokines treatment

Apart from studies on astrocyte gene profiles, part of the work described in this thesis focused on microglia. The main observation in this part was that while gene expression profiles were very similar among cultures of microglia derived from different donors, the changes triggered by pro-inflammatory cytokines differ widely among different cultures, for reasons not fully understood at present.

Microglia cultured under standardized conditions show abundant expression of the chemokines CCL2, CCL3 and CXCL8 that each plays key roles in glial cell migration. Receptors for these chemokines are expressed not only in microglia themselves, but also on astrocytes. Also pleiotrophin, a molecule with anti-apoptotic, mitogenic, angiogenic and chemotactic activities is abundantly expressed. Interestingly, CCL2 and pleiotrophin are also expressed at high levels in astrocytes suggesting that they are important mediators in the intercellular communication by glial cells (see also below). Along with pleiotrophin, that has distinct neuroprotective qualities, also the known neuroprotectants neuromodulin (GAP-43, a novel microglia gene), endothelin-2, and several members of the bone morphogenetic protein family (BMP2A, BMP2B and/or BMP4) are expressed at high levels by microglia. These molecules share a variety of activities that promote neuronal growth and neurite extension and mediate neural development. Expression of these genes involved in neuroprotection and regeneration indicates that microglia actively participate in protective functions in the CNS. This is consistent with the generally growing awareness that microglia are not just destructive cells that limit themselves to phagocytosing cell debris and pathogens, but are active also in promoting neuronal survival and repair.

As stated before, while these abundantly expressed genes are consistently found in different microglia cultures, the way these cultures respond to pro-inflammatory stimuli including TNF-α or IFN-γ turned out to be very different each time we performed an analysis. None of the differences appeared to correlate with known parameters of the donor samples used to generate the microglial cultures including post-mortem delay, age, sex or clinical history of the donor. Variations in the outcome of well-controlled experiments are obviously frustrating to researchers but in this particular case, we strongly believe that the variation in microglia responses is not the

result of experimental artifacts or methodological flaws, but reflects a real biological difference amongst the different cultures. Interestingly, similar experiences by others can be tracked in a few papers. Hua and Lee for example, also noted marked variation in chemokine responses to cytokine or LPS stimulation between human microglia cultures from different donors, but somehow did not show any data on this variation nor elaborated on the issue (61). In a study on the effects of the amyloid-beta peptide on human microglia gene profiles, the researchers pooled mRNA samples from five different human samples rather than analyze individual samples, possibly again to accommodate individual variations (62). Also in murine microglia variations occur in the response monitored in different preparations, as reported by Carson and colleagues in their attempts to identify microglial activation markers (63).

One factor that might help explain the above variations includes regional differences in microglia features. For example it has recently been reported that expression of the TREM-2 receptor on murine microglia differs quite markedly from one region of the brain to the next (64). Also other features of microglia may be differentially developed dependent on the architectural context of the cells. A challenge remains to understand this remarkable heterogeneity in human adult microglia gene profiles upon activation.

Communication between astrocytes and microglia

As explained above, the cell culture models for either astrocytes or microglia that we have examined in our studies do not really represent fully resting cells as one would expect to find in a normal healthy brain. Astrocytes as well as microglia are proliferating and they are stimulated not only by the mere act of culturing but also by serum-derived factors or exogenous growth factors such as GM-CSF. Their morphology is consistent with cells being taken to a certain state of activation. For astrocytes, this state may be comparable to the state of gliosis. Likewise, microglia in culture do not fully resemble the highly ramified resting microglia that are seen in normal brain tissue, but display clear signs of activation as well. In both cases, cells may be considered to reflect glial cells as they will behave immediately following trauma, infection or damage.

It is of interest to consider the mediators that according to our gene profiling studies now emerge as the most dominantly expressed gene products in either type of cell under these partially activated states. Figure 7.4 summarizes the soluble mediators that are amongst these dominant products (indicated by bold “production“ arrows) and separates them into two groups. The first is composed of mediators that are relevant to regulating growth and differentiation of surrounding neurons and endothelial cells, and

include well-known growth factors such as VEGF, GAP-43 and pleiotrophin. The second group includes primarily mediators of growth and migration for astrocytes and microglia themselves; receptors for these molecules are expressed by either type of glial cell (indicated by thin "receptor" arrows in Figure 7.4). Well-known mediators that emerge as dominant players in the cross-talk between astrocytes and microglia include IL-1β and TNF-α, consistent with a wealth of previous data on these key mediators. Microglia are particularly active in producing IL-1β while astrocyte are strong TNF-α producers. Yet, a few other equally dominant mediators emerged from our studies that are less well known as mediators of glial cell interaction. These include CCL2 (formerly known as MCP-1) that our data reveal as a dominant product of both cultured astrocytes and microglia, and for which the receptor is also expressed by either type of cell. Also bone morphogenetic protein 2A (BMP-2A) is amongst the mediators produced at high levels by both cell types. While BMPs are known to affect the developmental program of astrocytes, their effects on microglia remain to be established. Interestingly another BMP family member, viz. BMP2B and/or BMP4 (the gene probe on the array does not discriminate between these two) is a major microglia product indicating that BMPs are mediators in glial cell communication.

A final point that could be made when reflecting on the data that are summarized in Figure 7.4 is the notion that several chemokines are involved in the intercellular communication between astrocytes and microglia, along with being autocrine mediators. Chemokines are often regarded as molecules that primarily serve to recruit hematogenous lymphocytes and thus promote inflammation. Yet, it should be kept in mind that also glial cells themselves - more close neighbours - are very well capable of binding and responding to several chemokines (65). Several chemokines, especially CCL2, CCL3 and CXCL8, may thus well be primarily aimed at guiding local glial cell responses rather than recruit macrophages or T-cells.

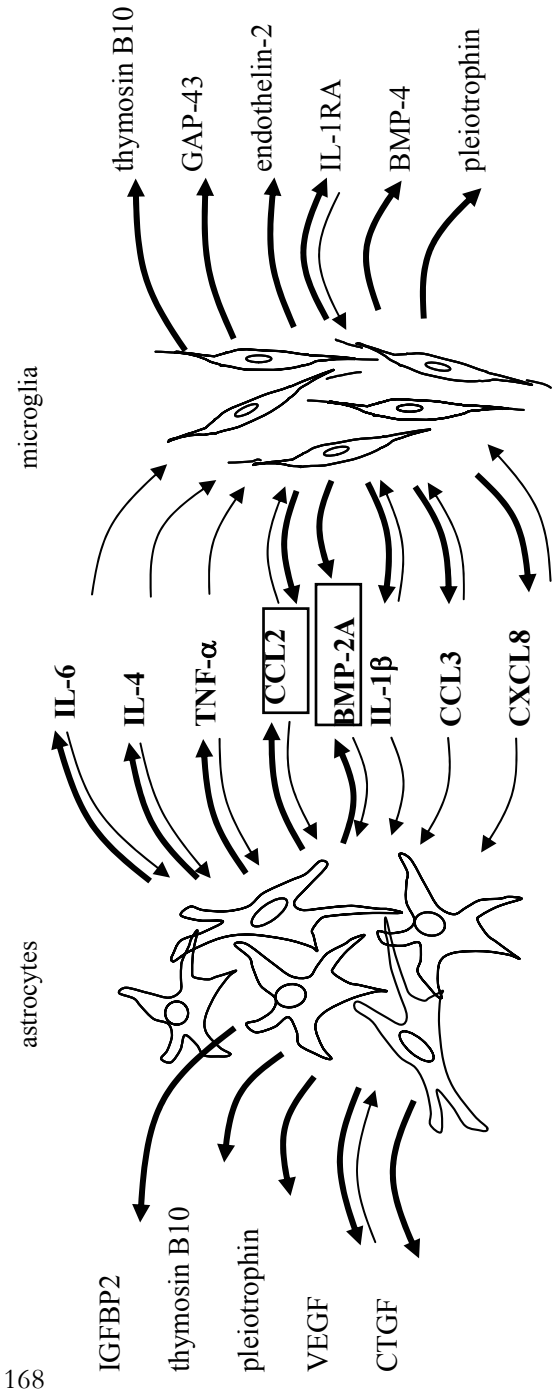

Figure 7.4. Key mediators in communication between human adult astrocytes and microglia

Possible role of HHV-6 in MS

In Chapter 1 of this thesis we discussed general mechanisms by which virus infection could conceivably promote an autoimmune response against myelin, and thus trigger or facilitate the development of MS. Such potential mechanisms include molecular mimicry and determinant spreading. To the best of our knowledge, only one report suggests a possibility for molecular mimicry to be operational in the case of HHV-6 by identifying a 7-amino acid peptide sequence in the U24 region of HHV-6 that is identical to the myelin basic protein sequence 96-102 (66). While cross-reactivity of T-cell clones could be demonstrated at the level of reactivity to synthetic peptides, no evidence exists to suggest that such cross-reactivity occurs at the biologically relevant level of reactivity to protein antigens. Also given other considerations, such as for example the widespread character of HHV-6 infection in humans, it seems unlikely that such cross-reactivity is relevant to understanding a possible involvement of HHV-6 in MS. Likewise, a mechanism of determinant spreading (viral infection triggers novel response cascades against autoantigens from the infected tissue) does not appear to be a likely one in the case of HHV-6. As explained before, our collective data on the impact of HHV-6 on astrocytes does not appear to be supportive at all to the idea that HHV-6 infection would spark a local inflammatory reaction, at least not after infecting astrocytes. Instead, the reverse is much more likely in that HHV-6 infection counteracts local inflammation.

HHV-6 infection of astrocytes clearly does not lead to production of factors that could activate T-cells such as IL-12 or high levels of TNF-α. One of the few pro-inflammatory mediators induced at all is CCL5 (RANTES) which may assist in recruitment of T-cells into the CNS. Yet, it should be kept in mind that the several different receptors for CCL5 are also expressed on glial cells themselves and the modest increase in CCL5 production in HHV-6-infected astrocytes may easily be absorbed by neighbouring cells before it could lead to levels of CCL5 that would attract T-cells from the blood. Even when an inflammatory reaction would start for another reason, the presence of HHV-6 in astrocytes would help down-regulate T-cell activity by triggering production of the anti-inflammatory mediators IL-10 and IL-11, as already elaborated above and in Chapter 4.

Apart from activating T-cells, HHV-6 infection could also lead to activation of microglia, which is the very first step in the formation of an MS lesion and a key element in its continued development. Potent activators of resting microglia including IL-1β, TNF-α and GM-CSF, however, are not induced in astrocytes by HHV-6 in any detectable way. Only when inflammation has already started (mimicked in our experiments by the addition of exogenous

TNF-α and IL-1β) increased production of IL-1β and GM-CSF was observed, a response that would further stimulate microglia activation and proliferation under such conditions. It should be noted, however, that at this stage microglia produce distinct neuroprotective mediators and no longer represent polarized destructive elements (67,68). Also (as noted before), the mediators IL-10 and IL-11 that are released by infected astrocytes under these conditions are known to down-regulate expression of the major co-stimulatory molecules CD40, CD80 and CD86 as well as MHC class II on microglia, impairing their ability to activate T-cells (56,57,58,59). Thus, our data suggest that HHV-6 infection of astrocytes will not easily lead to activation of surrounding microglia in resting tissue. When another factor has already triggered a local inflammatory reaction HHV-6-infected astrocytes can further promote microglia activation but at the same time inhibit their antigen-presenting ability.

More subtle ways for HHV-6 to influence pathogenic autoimmune responses in MS could include effects on apoptosis-induction pathways, a central mechanism for astrocytes to eliminate undesirable (autoimmune) T-cell activity from the CNS. This mechanism is important in protecting the CNS from immune-mediated damage. Human adult astrocytes express both CD95 (Fas) and CD95L (Fas ligand) both of which can induce apoptotic death in infiltrating T-cells by binding to their counterparts CD95L and CD95, respectively, that are expressed on T-cells. Upon infection by HHV-6, no change in astrocyte expression was observed of any of the apoptosis-related genes represented on the cDNA arrays including CD95, CD95L, Fas soluble protein Apo 1, secreted apoptosis related protein 1 (SARP1), SARP3 or apoptosis-related protein TFAR15. These genes were all expressed at low but detectable levels in uninfected astrocytes and their expression levels remained unchanged upon infection.

A recent paper by Dietrich and co-workers has suggested yet another way by which HHV-6 could influence the development of MS lesions and this is by interfering with repair. When oligodendrocyte precursor cells were experimentally infected with HHV-6, their proliferation and development was markedly impaired (49). If this would also be the case *in vivo*, an obvious effect could be interference with the natural process of myelin repair. An inhibiting effect of HHV-6 on the growth and development of such oligodendrocyte precursors is in line with our own observation that HHV-6 slows down growth in astrocytes as well. Yet in the case of astrocytes, diminished growth could be regarded as a positive side effect since reduced proliferation of astrocytes (gliosis) may in fact allow for more opportunity to repair myelin rather than turning a lesion into an eternally dysfunctional scar. In whatever way the net effect of HHV-6 of growth of

glial cells may work out *in vivo*, it still does not represent a likely mechanism that could explain a role of HHV-6 as an initial trigger for lesion formation in MS.

When taken together, our data and considerations give us little reason to strengthen any suspicion towards HHV-6 as an etiological agent in MS. Although we are far from fully understanding all effects that the virus may have on CNS functioning, the currently collected pieces of the puzzle all point to a more immune-regulatory effect of HHV-6 infection rather than a clear-cut pro-inflammatory effect, at least after infection of astrocytes. As reviewed above, also no clues were found for other and more subtle ways for the virus to promote T-cell mediated autoimmune reactions in the CNS, not even under pre-existing inflammatory conditions. The fact in itself that HHV-6 is a common infection whereas MS is not a common disease, does not necessarily rule out a contributing role of the virus. Like in the case of EBV which is also a common infection, multiple ways exist to accommodate a virus as a crucial co-factor in the pathogenesis of disease without viral infection being equally rare as the disease itself.

We showed that cultured human astrocytes and microlgia present a unique system to examine in detail their response to defined stimuli and virus infection. It is our conviction that this will help to understand the way glial cells behave in an intact brain under a variety of conditions. The major gene products in astrocytes and microglia cultured under standardized conditions are genes involved in growth processes. Microglia from different donors treated with pro-inflammatory cytokines show a widely diverse gene response in each individual culture. In addition the data as described in this thesis indicate that infection of astrocytes with HHV-6 is silent, the virus has no impact on the astrocyte gene profile. Furthermore, pro-inflammatory cytokine treatment of HHV-6-infected astrocytes resulted in an anti-inflammatory gene response. In conclusion all the data collected by us indicate that HHV-6 is highly unlikely to play a pathogenic role in the development of MS.

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Nederlandse Samenvatting

Virus infectie (met HHV-6) en het cytokinen profiel in menselijke hersencellen (glia cellen)

Doel van het onderzoek

Het ophelderen van de rol van het humane herpes virus 6 in de hersenen van de mens bij de ontwikkeling van multiple sclerose (MS)

MS is een chronische ontstekingsziekte van het centrale zenuwstelsel (CZS) met onbekende oorzaak, en waarvoor momenteel geen genezing bestaat. Het is de meest invaliderende ziekte van het CZS onder jong volwassenen, de ziekteverschijnselen worden meestal tussen de leeftijd van 20 en 40 jaar duidelijk. Bij MS patienten raakt de myeline, de witte stof in de hersenen en ruggenmerg die zorgt voor overdracht van zenuwprikkels, beschadigd door ontstekingen. MS gaat gepaard met vele ziekteverschijnselen, variërend van tinteling in de ledematen en tijdelijk slecht zien tot ernstige beperkingen in lichaamsbewegingen en tevens vermoeidheid en depressiviteit. Helaas ervaren mensen met MS nog vaak onbegrip uit hun omgeving, voortkomend uit de onbekendheid van de ziekte. Er is weinig bekend over het ontstaan van de ziekte. Door bestudering van de verspreiding van MS weet men dat zowel omgevingsfactoren als erfelijkheid de kans op MS beïnvloeden. Omgevingsfactoren zoals zonlicht, voeding en infectie met virussen of bacteriën kunnen naast het risico op MS ook het beloop ervan beïnvloeden. Virussen zijn beruchte micro-organismen. Ze leven in en vaak ten koste van andere organismen en passen zich snel aan. Virussen worden van oudsher verondersteld een rol te spelen bij het ontstaan en het beloop van MS. In de laatste decennia hebben vele onderzoeken de betrokkenheid van virussen bij MS ondersteund. Toch ontbreekt het nog aan inzicht over de precieze relatie tussen virussen en MS. Immers, de meeste virussen waarvan we vermoeden dat ze een rol spelen, zijn vrij normale virussen die het merendeel van de bevolking infecteren en allerminst specifiek zijn voor mensen met MS. Hoe kunnen deze virussen dan toch een rol spelen? Er is nog nauwelijks iets bekend over het gevolg van een infectie op het functioneren van hersencellen. Eén van de virussen die verondersteld worden een rol te spelen bij MS is humaan herpes virus-6 (HHV-6). Ook bij dit virus is het zo dat het niet specifiek is voor de ziekte en in de bevolking veel voorkomt, dus ook bij gezonde mensen. Toch wijzen sommige studies erop dat het virus vaker en actiever voorkomt in de hersenen van MS patiënten. Daarbij wordt vaak zonder meer verondersteld dat de aanwezigheid van het virus de ontstekingen verder stimuleert, maar harde gegevens hierover ontbreken. Mijn onderzoek was er op gericht om vast te leggen welk effect HHV-6 infectie nu precies heeft op menselijke hersencellen. Het is natuurlijk niet

mogelijk experimentele infecties met HHV-6 in de mens uit te voeren. Een goed alternatief lijkt het werken met gekweekte cellen uit hersenmateriaal, verkregen na het overlijden van donoren. Dit gebeurde in samenwerking met de Nederlandse Hersenbank. Hersencellen kunnen zelfs na de dood heel goed worden gekweekt door ze te voorzien van een kweekvloeistof met voedingsstoffen, voldoende zuurstof en een juiste temperatuur.
Wat valt er nu zoal te onderzoeken aan hersencellen in kweek? Gekweekte hersencellen kunnen blootgesteld worden aan zelfgekozen virusinfecties en verschillende ontstekingsbevorderende stoffen. (wat natuurlijk onmogelijk is bij proefpersonen). Op die manier is het dan mogelijk om in detail de effecten daarvan te leren begrijpen. Een groot voordeel van gekweekte hersencellen is dat er heel nauwkeurig, en per celtype afzonderlijk, gemeten kan worden welke stoffen geproduceerd worden. Een belangrijke groep stoffen die hersencellen maken is bijvoorbeeld de groep van ontstekingsstoffen. Door deze stoffen te maken en vanuit de cel de omgeving in te sturen kan een hersencel andere cellen beïnvloeden in hun groei, bewegingsrichting en andere activiteiten. Zulke stoffen noemen we cytokines en chemokines. Andere belangrijke stoffen die gemaakt worden kunnen zenuwcellen beschermen tegen schade, of een nabijgelegen bloedvatwand laten openen of juist sluiten. Een hersencel produceert dit soort stoffen niet voortdurend, maar pas als daarvoor de juiste prikkels worden ontvangen. Vaak is een hersencel daarbij gevoelig voor prikkels die gegeven worden door precies dezelfde stoffen die het zelf weer maakt om andere cellen te beïnvloeden. Zo ontstaat dus een netwerk van honderden stoffen die gezamenlijk en in wisselende samenstelling de ingewikkelde activiteiten van allerlei celtypen binnen het zenuwstelsel nauwkeurig sturen. Zo kan dan ook een passende reactie verzorgd worden op schade of infectie. Gegeven de ingewikkeldheid van dit soort boodschapper-netwerken is een goed begrip van de werking ervan afhankelijk van een meetmethode waarbij de aanmaak van vele stoffen tegelijkertijd kan worden gevolgd. In het onderzoek is daarom gebruik gemaakt van een zogenaamde cDNA array profileringstechniek. Bij deze techniek worden filters gebruikt waarop “malletjes” zitten voor het gelijktijdig registreren van de aanmaak van 268 verschillende stoffen. Genetisch materiaal (cDNA) uit de gekweekte hersencellen dat die aanmaak van stoffen weerspiegelt wordt radioactief gemaakt en op zo’n filter gebracht. Als de cel veel van dit radioactieve productie-materiaal oplevert, betekent dat vrijwel zonder uitzondering dat er ook veel van die bepaalde stof wordt gemaakt. De honderden verschillende “mallen” op de filter kunnen op deze manier gebruikt worden om van even zovele stoffen de productie nauwkeurig te meten. Een volledig overzicht, ook wel expressieprofiel genoemd, is dan het resultaat.

De menselijke hersenen en ruggenmerg bevatten twee belangrijke celtypen, neuronen (zenuwcellen) en glia cellen. Neuronen zijn betrokken bij informatie overdracht zowel binnen het CZS als in communicatie met de rest van het lichaam. Glia cellen ondersteunen en voeden neuronen en doen ook mee in bepaalde informatie-circuits. Daarnaast spelen glia cellen een heel belangrijke rol in het constant houden van het interne milieu van hersenen en ruggenmerg, en het regelen van bloedvatfuncties in het CZS. Daarbij hoort ook het regelen van ontstekingsreacties die als regel volgen op infecties, zuurstoftekort of andersoortige schade. Glia cellen zijn onder te verdelen in astrocyten, microglia en oligodendrocyten. In dit proefschrift is gebruik gemaakt van gekweekte menselijke astrocyten (hoofdstuk 2 t/m hoofdstuk 5) en gekweekte menselijke microglia (hoofdstuk 6). Astrocyten zijn de meest voorkomende cellen in het CZS. Ze zijn vooral betrokken bij het ondersteunen en voeden van andere cellen, ze communiceren direct met zenuwcellen en bloedvaten en na schade –zoals na een ontsteking tijdens MS- zijn ze primair verantwoordelijk voor de vorming van litteken weefsel. Microglia zorgen in eerste instantie voor het bevechten van infecties en het opruimen van restmateriaal dat ontstaat bij schade, celdood of ontstekingen. Ze spelen daarnaast ook een aanvullende rol in herstelprocessen.
In hoofdstuk 2 is onderzocht of de hierboven beschreven cDNA array techniek geschikt is voor het meten van stoffen in gekweekte menselijke astrocyten. Dit is eerst gedaan met cellen in een normale kweek, en vervolgens met cellen die geactiveerd werden door een paar van de meest belangrijke signaalstoffen voor ontstekingen. Deze signaalstoffen zijn eerder door anderen gevonden in de ontstekingshaarden van MS en het is daarom bekend dat ermee een redelijke nabootsing kan worden verkregen van het actieve milieu van zulke haarden. In beide situaties is met de cDNA array techniek de aanmaak van een groot aantal boodschapperstoffen door astrocyten gevolgd. De array techniek bleek prima geschikt te zijn voor de beoogde metingen. Wanneer een populatie astrocyten afkomstig van één donor op verschillende tijdstippen in kweek werd genomen en geanalyseerd op hun productieprofiel, was het resultaat steeds hetzelfde. Ook wanneer astrocyten uit hersenen van andere donoren werden gekweekt werd wederom hetzelfde profiel gemeten, ten teken dat de kweek- en meetmethode voldoende stabiel en betrouwbaar was en dat astrocyten uit de hersenen van verschillende donoren sterk op elkaar lijken. Met de methode werd vervolgens in kaart gebracht welk van de honderden verschillende stoffen nu precies reageerden op de ontstekingssignalen. Van veel stoffen was de productie sterk verhoogd, van sommige stoffen ging de productie juist omlaag. Hoofdstuk 2 geeft een compleet overzicht van alle reacties. Echt interessant werd het natuurlijk toen ook het virus erbij werd betrokken.

In hoofdstuk 3 en 4 is onderzocht wat nu precies het effect is van HHV-6 infectie op astrocyten. In hoofdstuk 3 is vastgesteld op welke manier zoveel mogelijk astrocyten in kweek konden worden geïnfecteerd. Daarbij werd onderzocht welke hoeveelheid van het virus het meest geschikt was, en hoe lang gewacht moest worden voordat effecten zichtbaar zijn. Na het testen van allerlei variaties bleek het maximaal haalbare een benadering waarbij na 2 dagen ongeveer eenderde van alle astrocyten met HHV-6 geïnfecteerd was. Dit was eenvoudig zichtbaar omdat geïnfecteerde astrocyten met elkaar versmelten tot één grote reuzencel met vele kernen waarin viraal eiwit zichtbaar is. Langer wachten dan 2 dagen, of een grotere hoeveelheid virus leidde er slechts toe dat de astrocyten snel doodgingen, hetgeen uiteraard niet de bedoeling was. In hoofdstuk 4 is het effect van zo'n experimentele infectie op astrocyten bepaald, met gebruikmaking van de cDNA array techniek. Verrassend was dat na infectie van de astrocyten er helemaal niets veranderde aan de productie van de honderden stoffen gemeten met de array. Klaarblijkelijk weet het virus de astrocyt te infecteren zonder dat de cel een signaal afgeeft dat er iets aan de hand is. Het is goed denkbaar dat dit vermogen van HHV-6 tot "sluipmoord" de doorslag geeft bij zijn succes om bij veel mensen het CZS te infecteren zonder direct kwalijke gevolgen. Het virus bleek nog verder te gaan. Wanneer er ontstekingssignalen werden toegevoegd aan de geïnfecteerde astrocyten, net zoals eerder was gedaan met de gewone gezonde astrocyten, bleken de geïnfecteerde astrocyten heel anders te reageren. Veel meer beschermende en herstellende stoffen werden aangemaakt dan in gezonde cellen, en zelfs een paar uitzonderlijke stoffen die ontstekingen krachtig remmen. Hoewel de interpretatie van dit alles niet eenvoudig is zonder nog meer proeven te doen, lijkt het profiel dat werd gemeten heel sterk te wijzen op de mogelijkheid dat de HHV6 infectie van astrocyten veel eerder een ontsteking in het CZS afremt dan bevordert.
Hoofdstuk 5 (het laatste hoofdstuk gewijd aan astrocyten) beschrijft een serie verkennende proeven naar het voorkomen van Toll-like receptoren (TLR) in reactie op een HHV-6 infectie. TLR zijn eiwitten die we vanaf de geboorte hebben, ze zitten op het oppervlak van cellen om binnendringende virussen en bacteriën te herkennen. Er zijn in de mens tenminste tien van dergelijke TLR beschreven en op dit moment wordt er wereldwijd veel onderzoek gedaan naar hun werking. Meestal worden TLR onderzocht in weefsels die speciaal met afweer te maken hebben, zoals lymfeklieren en milt. Welke van de tien TLR in het CZS van belang zijn, en of ze betrokken zouden kunnen zijn bij herkenning van HHV-6 zijn de eerste vragen die opkomen. In de eerste proeven is gevonden dat HHV-6 infectie van astrocyten leidt tot verhoging van de aanmaak van sommige TLR. Dit zou men wellicht verwachten als een reactie op infectie, aannemend dat die

bepaalde TLR dan ook het virus herkennen. Daar bleek evenwel nog niets van. De signalen die astrocyten afgeven zodra TLR worden geactiveerd traden namelijk niet op. De vraag blijft dus of het virus überhaupt wel door TLR kan worden opgepikt en, als dat niet zo zou zijn, waarom de astrocyte dan de moeite neemt TLR verhoogd te produceren in reactie op het virus. Het is mogelijk dat het virus methoden heeft om binnen de cel de signalen van een TLR te blokkeren, en zo afweerreacties te saboteren.

Hoofdstuk 6 gaat over een ander belangrijk celtype in het CZS, de microglia. Microglia zijn lastiger te bestuderen omdat ze niet goed houdbaar zijn en alleen direct na hun zuivering uit hersenweefsel onderzocht kunnen worden. Anders dan astrocyten kunnen ze niet bevroren bewaard worden. Allereerst werd met de cDNA profileringsmethode bekeken of microglia uit verschillende donorhersenen een stabiel expressieprofiel hebben. Dat bleek inderdaad het geval. Opmerkelijk was dat microglia gestimuleerd met ontstekingsstoffen die ook bij de astrocyt waren toegepast, sterk wisselende profielen hadden. Microglia uit verschillende bronnen gaven verschuivingen in het profiel van de honderden stoffen. Momenteel blijft dit wisselvallige gedrag van microglia wat raadselachtig, maar in eerder gepubliceerde resultaten van andere onderzoekers vonden we aanwijzingen dat ook zij nogal eens geplaagd zijn door wispelturige microglia. Samen met de overtuiging dat de meetmethode geen bron van de wisselende resultaten kan zijn, gaan we er van uit dat microglia inderdaad nogal wisselend gedrag kunnen vertonen wanneer ze geprikkeld raken. Dit heeft mogelijk te maken met de precieze plaats in de hersenen waaruit de cellen zijn gezuiverd (was niet voor elke proef dezelfde), of met klinische variabelen van de donoren. Met dit wisselend microglia gedrag dient in toekomstig werk rekening gehouden te worden. Het maakt het werken met gekweekte microglia een stukje lastiger dan het werken met astrocyten, maar zeker zo interessant.

In hoofdstuk 7 (algemene discussie) worden de resultaten kort beschouwd en met elkaar in verband gebracht. Natuurlijk zijn er nog geen eindconclusies te trekken. Wanneer is onderzoek helemaal af? De vraag of HHV-6 een rol speelt in MS blijft open, maar de resultaten uit dit proefschrift geven aan dat niet voetstoots mag worden aangenomen dat infectie met HHV-6 de ziekte zou verergeren. Integendeel, HHV-6 lijkt veel meer een remmende factor te zijn wanneer het in een ontstekingshaard zit. Effecten kunnen anders zijn bij infectie van andere celtypen. Met gekweekt menselijk hersenmateriaal is dit verder te onderzoeken, maar bij gebruik van gekweekte microglia is dus wel enige voorzichtigheid geboden, gezien het wispelturige karakter van deze cellen. Maar juist dit karakter, en dat van andere celtypen in de menselijke hersenen blijkt zeer toegankelijk te zijn met de nu verder ontwikkelde onderzoeksmethoden.

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Sonja Meeuwsen

Curriculum Vitae

Sonja Meeuwsen was born on the 6th of February 1976 in Valkenswaard. In 1994 she finished her secondary school at the "Hertog Jan College" in Valkenswaard, the Netherlands. From 1994 till 1999 she studied Medical Biology at the University of Amsterdam. During this study she obtained research experience at the department of clinical immunology at the Academic Medical Center in Amsterdam. She participated in asthma research focused on cytokine measurements in blood and in bronchial alveolar lavage fluid of patients. Furthermore, she did a social policy study for the Dutch rheumatic foundation, in the context of her specialization "Social biology".
In April 2000 she started her PhD, a collaboration between the free university of Amsterdam (department of cell biology), TNO Quality of Life (department Biomedical Research) in Leiden and the Netherlands Brain Bank in Amsterdam. The findings of her study are described in this thesis.